

DTIC FILE COPY

(1)

AD _____

AD-A219 311

NEW APPROACHES TO ATTENUATED HEPATITIS A VACCINE DEVELOPMENT:
CLONING AND SEQUENCING OF CELL-CULTURE ADAPTED VIRAL cDNA

FINAL REPORT

Stanley M. Lemon, M.D.

October 1, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-85-C-5272

University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-7030

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings contained in this report are those of the author and should not be construed as an official Department of the Army position unless so designated by other documentation.

90 03 08 079

DTIC
ELECTE
MAR 03 1990
S

REPORT DOCUMENTATION PAGE

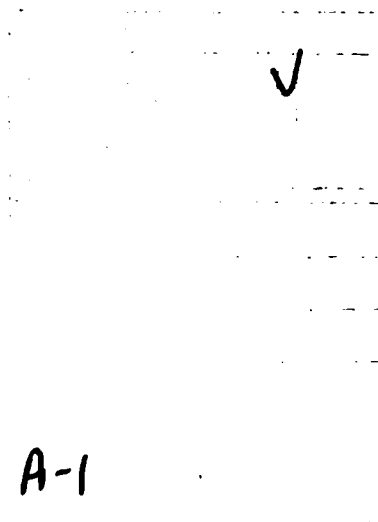
Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			7a. NAME OF MONITORING ORGANIZATION		
6a. NAME OF PERFORMING ORGANIZATION University of North Carolina		6b. OFFICE SYMBOL (If applicable)	7b. ADDRESS (City, State, and ZIP Code)		
6c. ADDRESS (City, State, and ZIP Code) Chapel Hill, North Carolina 27599-7030			9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-85-C-5272		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (If applicable)	10. SOURCE OF FUNDING NUMBERS		
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012			PROGRAM ELEMENT NO. 61102A	PROJECT NO. 3M1- 61102BS10V	TASK NO. AA WORK UNIT ACCESSION NO. 055
11. TITLE (Include Security Classification) New Approaches to Attenuated Hepatitis A Vaccine Development: Cloning and Sequencing of Cell-Culture Adapted Viral CDNA					
12. PERSONAL AUTHOR(S) Stanley M. Lemon, M.D.					
13a. TYPE OF REPORT Final Report		13b. TIME COVERED FROM 9/15/85 - 3/14/89	14. DATE OF REPORT (Year, Month, Day) 1989 October 1		15. PAGE COUNT 48
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Hepatitis A Vaccine; Molecular Cloning and Hybridization; Strain Differences; Attenuation; RA I (K T) ←		
06	13				
06	03				
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
<p>— To determine the molecular changes associated with adaptation of hepatitis A virus (HAV) to growth in cell culture, the genome of a cell culture-adapted variant of HM175 strain HAV (p16 HM175, 16th in vitro passage level) was molecularly cloned and the complete nucleotide sequence of the virus determined. This virus is well adapted to growth in cell culture, but remains virulent as shown in monkey challenge experiments. p16 HM175 was found to contain a total of 19 mutations from the wild-type genome, including five mutations in the 5' nontranslated region, one mutation in the 3' nontranslated region, and 13 mutations predicting 8 changes in the amino acid sequences of HAV proteins. In an effort to assemble an infectious cDNA clone, partial inserts from ten HM175 cDNA clones were assembled into a single construct containing the consensus p16 HM175 sequence. Flanking homopolymeric dC-dG tails derived from the original cloning procedure were removed, and the full length sequence of p16 HAV cDNA was</p>					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input checked="" type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Virginia M. Miller			22b. TELEPHONE (Include Area Code) 301/663-7325		22c. OFFICE SYMBOL SGRD-RMI-S

19. Abstract continued

inserted between the HindIII and XbaI sites of the transcription vector pGEM3. At the conclusion of the contract, the infectivity of this construct (pHAV/pl6) was under evaluation. The sequence of the P1 genomic regions of two plaque-purified, cytopathic variants of HM175 virus was determined from virion RNA; one of these variants was shown to be a spontaneous neutralization escape mutant. -A novel immunoaffinity-linked nucleic acid amplification system (antigen-capture/polymerase chain reaction, or AC/PCR) capable of the strain-specific detection of HAV in clinical specimens was developed and evaluated. Molecular cloning and partial sequencing of the genome of PA21 strain HAV was undertaken in an effort to determine the extent of genetic divergence from human HAV.

... to ...



A-1



SUMMARY

Research effort under this contract has centered on the determination of molecular changes associated with adaptation of hepatitis A virus to growth in cell culture, and with attenuation of virus virulence. To determine the molecular changes associated with adaptation of hepatitis A virus (HAV) to growth in cell culture, the genome of a cell culture-adapted variant of HM175 strain HAV (p16 HM175, 16th in vitro passage level) was molecularly cloned and the complete nucleotide sequence of the virus determined. This virus is well adapted to growth in cell culture, but remains virulent as shown in monkey challenge experiments with virus at the 22nd passage level (see below). p16 HM175 was found to contain a total of 19 mutations from the wild-type genome, including five mutations in the 5' nontranslated region, one mutation in the 3' nontranslated region, and 13 mutations predicting 8 changes in the amino acid sequences of HAV proteins. Only one amino acid substitution occurred among the capsid proteins (VP2), while others involved proteins 2A, 2B, 2C, VPg and 3Dpol. The distribution of these mutations within the genome suggests that changes in RNA replication may be of primary importance in adaptation of the virus to growth in vitro.

An infectious cDNA clone derived from virulent HAV would be of considerable value in experiments designed to determine the molecular basis of attenuation. In an effort to construct such a clone, cDNA fragments from ten HM175 clones were assembled into a single construct, flanking dC-dG tails derived from the original homopolymeric cloning procedure were removed, and the full length sequence of p16 HAV cDNA was inserted between the HindIII and XbaI sites of the plasmid vector pGEM3 to create pHAV/p16. At the conclusion of the contract, the infectivity of this construct was under evaluation.

The pHM175 variant of HAV, recovered by disruption of persistently infected continuous green monkey kidney (BS-C-1) cells, induces a cytopathic effect during serial cell culture passage. Virus neutralization and epitope-specific radioimmunofocus assays showed that pHM175 virus stock contained two virion populations, one with altered antigenicity including neutralization-resistance to monoclonal antibody K24F2, and the other with normal antigenic characteristics. Both virus types were cytopathic in monkey kidney cell cultures, and both displayed a rapid replication phenotype when compared with the parental virus used to establish the original persistent infection (p16 HM175 virus). Within the P1 genomic region, amino acid replacements were present in K24F2⁻ virus (43c clone) at residue 90 of VP3 and residues 197 and 276 of VP1; changes in K24F2⁺ virus (18f clone) were at residues 91 of VP3 and 271 of VP1. These mutations did not reduce the thermal stability of the virion. Although cytopathic viruses do not share a unique capsid mutation, the emergence of an antigenic variant during persistent infection suggests that changes in capsid proteins may influence virus replication in cell culture.

A cell culture-adapted, neutralization-resistant variant of HAV (HM175/S18), derived from p16 virus at the 22nd in vitro passage level, was examined for virulence in the New World owl monkey model. Monkeys challenged with approximately 5×10^4 RFU of HM175/S18 developed liver enzyme elevations and virologic markers of infection resembling those previously noted following

wild-type virus challenge. Fecal shedding of viral antigen may have been less following challenge with the S18 virus than with wild-type virus, however. Virus shed in the stools of monkeys challenged with HM175/S18 was found to have reverted to neutralization susceptibility, although double antibody radioimmunofocus assays provided solid-evidence for replication of the mutant virus in the monkeys.

A novel immunoaffinity-linked nucleic acid amplification system (antigen-capture/polymerase chain reaction, or AC/PCR) was developed for detection of HAV in clinical specimens. Immunoaffinity capture of virus, synthesis of viral cDNA, and amplification of cDNA by a polymerase chain reaction (PCR) were carried out sequentially in a single tube. This approach simplified sample preparation and significantly enhanced the specificity of conventional PCR. AC/PCR consistently detected less than one cell culture infectious unit of virus in 80 ul of sample. Sequencing of AC/PCR reaction products from 13 HAV strains demonstrated remarkable conservation at the nucleotide level, but suggested the presence of distinct virus genotypes circulating within geographically defined regions. Virus strains recovered from two military epidemics of hepatitis A were identical in sequence, providing evidence for a previously unrecognized epidemiologic link between these outbreaks.

PA21, a strain of HAV recovered from a naturally infected captive owl monkey, is antigenically indistinguishable from human HAV although cDNA-RNA hybridization has suggested a significant sequence divergence. The nucleotide sequence of the P1 region of the PA21 genome was found to have only 83.2% identity with HM175 virus, a difference approximately twice as great as that found between any two previously studied human strains. Most nucleotide changes were in third base positions, and the amino acid sequence of the capsid proteins was largely conserved. Similar genetic divergence was evident in P2 and P3 genomic regions, but the 5' and 3' noncoding regions were relatively conserved (89.2% and 93.7% nucleotide identity respectively). These data indicate that PA21 virus represents a unique genotype of HAV and suggest the existence of an ecologically isolated niche for HAV among feral owl monkeys.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

TABLE OF CONTENTS

Report Documentation Page (DOD1473)

Title Page-----	1
Summary-----	2
Forward-----	4
Table of Contents-----	5
Introduction-----	6
Experimental Approach-----	9
Research Progress-----	10
Conclusions-----	24
Tables and Figures-----	29
Literature Cited-----	40
Publications -----	46
Personnel-----	48

INTRODUCTION

Background Hepatitis A virus (HAV) is a human picornavirus with a worldwide distribution (Lemon, 1985). It poses a considerable threat to military forces of the United States due to its capacity for epidemic spread, and the fact that acute hepatitis A associated with primary infection with HAV is a protracted illness with a prolonged convalescence. At present, there is a low prevalence of antibody among active duty U.S. forces, indicating a high degree of susceptibility.

No vaccine is currently available for prevention of infection with this medically important virus. Three general approaches to HAV vaccines have been considered, including development of an inactivated, cell-culture derived vaccine (Binn et al., 1985), an attenuated vaccine derived by extensive passage of virus in vitro (Provost et al., 1982; Provost et al., 1986), and more novel vaccines based on synthetic peptide or recombinant DNA technology. A detailed discussion of these approaches, all of which are beset with difficulties may be found in Report Number 1 of this contract, or in the review by Lemon (1985). This contract has focused on understanding the molecular basis of attenuation of HAV, as such an understanding might open new approaches to development of an economic and effective HAV vaccine.

Molecular basis of cell culture adaptation and attenuation of HAV

Although attenuation of HAV has been shown to result from extended in vitro passage of virus and has been proposed as an approach to the development of attenuated vaccines (Provost et al., 1982; Provost et al., 1986). Wild-type HAV, recovered from feces or liver of infected primates, replicates slowly and to low titers in cell culture. Most strains of HAV do not induce cytopathic effects in cell cultures, and persistent infection is characteristic of this virus system. With successive passages, however, the virus becomes progressively adapted to growth in vitro (Binn et al., 1984; Frösner et al., 1979; Provost and Hilleman, 1979). Adaptation to growth in cell culture results in a shortening of the interval between inoculation of cultures and maximum virus yield as well as increases in the yield of virus, and appears to be a multi-step process. Passage of virus in vitro has also been associated with attenuation of virulence as evidenced by a reduction in the ability of the virus to induce liver injury in both experimentally challenged primates and man (Provost et al., 1982; Karron et al., 1988). Extensively passaged virus may even be incapable of replication in vivo, as no clinical evidence of infection and no measurable antibody response has followed parenteral challenge of humans with very extensively passaged virus (Provost et al., 1986). However, because some virus variants that are well adapted to growth in cell culture retain nearly unaltered virulence in some primate species (e.g., owl monkeys) (Lemon et al., 1987a; Lemon et al., 1989), attenuation and cell culture adaptation represent separate although closely related phenotypic characteristics of HAV.

The molecular mechanisms underlying either adaptation of HAV to growth in cell culture or attenuation of HAV remain unknown. Adaptation of HAV to growth in cell culture results in profound changes in the biologic characteristics of the virus and with continued passage of virus has been associated with a reduction in virulence in several different species of primates. A

primary effort under support of this contract has therefore been the cloning and sequencing of the genome of a cell culture-adapted variant of HM175 strain HAV, and the identification of mutations in this virus that were associated with adaptation of this virus to growth in vitro. The HAV variant studied (p16 HM175 virus), had been passaged a total of 16 times in cell culture. We believe it to be nearly fully virulent, given the results of monkey challenge experiments with a neutralization escape variant derived from p16 HM175 which was studied at the 22nd in vitro passage level (see below).

Infectious HAV cDNA Although construction of an infectious HAV cDNA clone has been accomplished by other investigators, the only available infectious clone is derived from an attenuated virus variant (Cohen et al., 1987c). An infectious clone derived from virulent HAV is not available, but would be of considerable value in experiments designed to determine the molecular basis of attenuation. In addition, a virulent infectious clone would allow the construction of deletion mutants (e.g., in the 5' noncoding region) which could be examined for potential attenuation. Thus, the availability of an infectious cDNA clone derived from a virulent, but cell culture-adapted, HAV variant would open new avenues for development of candidate attenuated vaccine variants.

Virulence of a neutralization escape variant of HAV We have considered the possibility that selection of virus variants for resistance to neutralizing monoclonal antibody might be associated with attenuation of virus. Neutralization escape variants derived from a number of other viruses, including some picornaviruses, have been shown by other workers to be relatively attenuated. We selected a number of variants of the HM175 strain HAV for resistance to murine monoclonal neutralizing antibodies (Stapleton, 1987). These variants of HAV were selected in an effort to further define the molecular structure of the major neutralization epitopes of the virus. Individual variants demonstrate resistance to each of an extended panel of neutralizing monoclonal antibodies (Ping et al., 1988). Neutralization resistance is related to reduced binding of monoclonal antibodies, due to mutations at residue 70 of capsid protein VP3 or residue 102 of capsid protein VP1 (Ping et al., 1988). Despite this, the variants grow well in vitro and are detectable in conventional immunoassays utilizing polyclonal antisera.

The fact that there is no epidemiologic or clinical evidence to suggest the evolution of antigenic variants of HAV in vivo suggests the existence of tight biologic constraints on the degree of variation permissible in the immunodominant epitopes of the virus. These constraints do not appear to be operative in vitro. One possible explanation is that the conserved antigen represents part of the hepatocyte receptor-binding domain on the virion surface. It was of considerable interest, therefore, to determine whether such variant viruses remain infectious or have altered pathogenicity in primate hosts. Accordingly, we initiated a collaborative study with COL J. LeDuc, Ph.D. of USAMRIID and L. Binn, Ph.D. of WRAIR in an effort to determine whether the S18 variant of HM175 virus, selected for high level resistance to monoclonal K24F2, is attenuated for New World owl monkeys, and whether infection of the owl monkey with this virus is associated with reversion of neutralization resistance.

Cytopathic HAV variants Recently, several unique variants of HAV have been reported to induce a cytopathic effect in monkey kidney cells in vitro (Venuti et al., 1985; Anderson, 1987a; Cromeans et al., 1987; Nasser and Metcalf, 1987). In at least three instances, cytopathic variants have emerged during continued passage of persistently infected cell cultures (Anderson, 1987a; Cromeans et al., 1987; Nasser and Metcalf, 1987). An important attribute of these cytopathic variants, each derived from the HM175 strain of HAV, is that they appear to be uniquely well adapted to growth in cell culture. One such variant (pHM175, Cromeans et al., 1987) replicates significantly more rapidly than its noncytopathic precursor, with maximum viral yields reached as early as 40 hours after inoculation of cells under one-step growth conditions (Cromeans et al., 1989). In further characterizing this cytopathic variant, we noted that the virus had spontaneously acquired a mutation within an immunodominant neutralization epitope (Ping et al., 1988). This finding prompted a detailed antigenic and genetic analysis of each in an effort to better understand the molecular basis for their unique growth properties in cell culture.

Identification of specific strains of HAV The development of a relatively rapid and specific technique for identifying specific strains of HAV was a major aim of the original contract application, as clinical trials of live and inactivated HAV vaccines will require the availability of methods capable of distinguishing wild-type HAV strains from vaccine virus. Certain differentiation between HAV strains has been accomplished only by "RNA fingerprinting", or by a comparison of nucleotide sequences derived from molecularly cloned viral cDNA (Weitz and Siegl, 1985; Ticehurst et al., 1989). Such approaches require considerable quantities of virus or the adaptation of virus to growth in cell culture, in itself a difficult task and one which may lead to mutations within genomic RNA. These labor intensive methods of distinguishing between viral strains are not directly applicable to the small quantities of virus usually present in clinical specimens, and have thus been of limited use in epidemiologic studies. To overcome these limitations and develop a molecular approach to studying the epidemiology of HAV, we turned to the polymerase chain reaction (PCR) as a means of amplifying viral nucleic acid present in clinical specimens.

PCR involves the selective, enzymatic amplification of a segment of a DNA molecule and may effect a greater than 10^6 -fold enrichment of a target DNA sequence (Saiki et al., 1988). The exquisite sensitivity of this procedure makes it an attractive approach to viral diagnosis, but also leads to potential problems with respect to specificity. Contamination of clinical specimens with miniscule quantities of recombinant nucleic acid present in the laboratory environment may lead to false-positive PCR results, and in some cases random priming of complex mixtures of non-specific nucleic acids may lead to amplification of stochastic reaction products. Moreover, typical methods for preparing viral nucleic acid for PCR are tedious, involve multiple manipulations, and are a potential source of contamination. To reduce these potential problems, we developed an integrated immunoaffinity/PCR procedure (antigen capture/polymerase chain reaction assay, or AC/PCR) for selective amplification of antigen-associated viral nucleic acid. This was accomplished by a solid-phase antigen-capture step, carried out in monoclonal antibody-coated microcentrifuge tubes, followed by heat denaturation of immobilized

virus, polymerase chain amplification of reverse-transcribed cDNA and primer-extension sequencing of amplified transcripts.

Simian PA21 strain of HAV In 1980, during collaborative studies between the WRAIR and the Gorgas Memorial Institute in Panama, an HAV strain (PA21) was recovered from a feral owl monkey shortly after its capture and admission to a primate holding facility in Panama (Lemon et al., 1982; Binn et al., 1984). Serologic testing of other owl monkeys demonstrated wide-spread infection with HAV within the colony during the preceding years, with most wild-caught animals seroconverting within months of capture. Virus recovered from infected monkeys was found to be antigenically indistinguishable from human HAV by quantitative in vitro neutralization assays (Lemon and Binn, 1983a), and in preliminary studies with monoclonal antibodies (Lemon et al., 1987b). Yet, slot-blot cDNA-RNA hybridization with probes derived from human HAV suggested that the PA21 virus is distinct from human HAV isolates (Lemon et al., 1987b). We molecularly cloned and partially sequenced the genome of PA21 virus, in an effort to determine the basis of its genetic diversity and how this might relate to its highly conserved antigenic structure.

EXPERIMENTAL APPROACH

1. We determined the complete nucleotide sequence of HM175 virus at the 16th in vitro passage level, and compared the sequence of this virus to that reported previously for its wild-type parent (Cohen et al., 1987a). These studies have determined that adaptation of this virus to growth in vitro was associated with a limited number of mutations, and have suggested a possible molecular basis for attenuation of the virus.

2. HAV cDNA from ten plasmid clones derived from either pl6 HM175 (Jansen et al., 1987) or wild-type HM175 (Ticehurst et al., 1983) were assembled into a genomic-length construct. This construct (pHAV/pl6) contains the complete consensus sequence of pl6 HM175, minus flanking homopolymeric tails, inserted into the transcription vector pGEM3. At the conclusion of this contract, this clone was being evaluated to determine whether or not it was infectious following transfection as either DNA or RNA into permissive cells.

3. We characterized a highly cell culture-adapted, cytopathic variant of HAV (pHM175 virus), and plaque-purified viruses that were representative of two virion populations present within pHM175 virus harvests. One of these viruses was found to be a spontaneous neutralization escape mutant. Both mutant (43c virus clone) and antigenically normal pHM175 virus (18f virus clone) were found to be cytopathic in BS-C-1 and FRhK-4 cells. We identified capsid mutations in both viruses by direct sequencing of virion RNA, and determined the stability of both viruses during short exposures to elevated temperatures.

4. We evaluated a cell culture-adapted, neutralization-resistant variant of HM175 strain HAV (HM175/S18) for hepatovirulence in seronegative owl monkeys. Approximately 5.3×10^4 radioimmunofocus-forming units of pl HM175/S18 were given intravenously to 6 susceptible owl monkeys. Outcome was

compared with that of 5 animals infected previously with wild-type (wt) HM175. Hepatovirulence of HM175/S18 was associated with reversion to the wild-type neutralization-susceptible phenotype.

5. We developed and evaluated an antigen-capture/polymerase chain reaction method for the strain-specific identification of HAV in human fecal samples, and applied this method to the partial nucleotide sequencing of a series of epidemiologically distinct human HAV strains.

6. The genome of PA21 strain HAV, a virus recovered from infected owl monkeys, was molecularly cloned and partially sequenced. The marked sequence divergence found between this virus and other strains of HAV recovered from humans suggests that PA21 virus is a unique genotype of HAV and may possibly be an indigenous virus of the owl monkey.

RESEARCH PROGRESS

I. Cloning and sequencing of p16 HM175 virus

p16 HM175 virus For analysis of the genetic changes accompanying adaptation of HAV to growth in vitro, we determined the sequence of HM175 virus at the 16th in vitro passage level, and compared its sequence with that of wild-type virus. This virus was initially isolated in primary African green monkey kidney cells (AGMK) from an homogenate of liver tissue obtained from a marmoset inoculated with wild-type HM175 strain HAV (6th marmoset-passage), a gift of S.M. Feinstone and R.H. Purcell, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA. The origin of this strain of HAV has been described (Gust et al., 1985). Virus was adapted to growth in vitro as described previously (Binn et al., 1984), and after 10 AGMK passages was passed once in continuous green monkey kidney cells (BS-C-1). Virus was then twice plaque-purified (Lemon and Jansen, 1985), and amplified at the sixteenth in vitro passage level (p16 HM175 virus) for isolation of viral RNA. Wild-type HM175 virus, employed for studies determining the efficiency of wild-type virus replication in vitro, was recovered from the feces of a New World owl monkey (*Aotus trivirgatus*) inoculated intravenously with a human fecal suspension containing HM175 strain HAV (LeDuc et al., 1983).

in vitro replication competence of p16 HM175 The efficiency of replication of p16 HM175 virus in BS-C-1 cells was compared with that of wild-type virus by determining the proportion of virus particles capable of inducing foci of infection detectable under agarose overlays 2 weeks after inoculation of cell cultures (for details, see Report Number 2). The wild-type virus used for this experiment was a suspension of feces obtained from an owl monkey (*Aotus trivirgatus*) experimentally infected with HM175 virus from human stool (LeDuc et al., 1983). The number of RNA-containing particles was estimated in both wild-type and p16 cell culture inocula by quantitative cDNA-RNA hybridization (Jansen et al., 1985). Foci of viral replication, identified by staining infected cell sheets with radiolabelled polyclonal human antibody (Lemon and Jansen, 1985), were considerably larger in cells inoculated with p16 virus than in cells inoculated with wild-type virus, indicating more efficient spread of the p16 virus to adjacent cells. Moreover, cDNA-RNA

hybridization results (not shown) indicated that the pl6 virus inoculum contained approximately 58 genome copies (i.e., RNA containing particle(s) per RFU, compared with 2.4×10^7 particles per RFU in the wild-type virus inoculum. Thus, there was an approximately 4000-fold difference in the ability of the cell culture-adapted (pl6) virus and wild-type virus to initiate foci of replication in BS-C-1 cells.

Molecular cloning of pl6 HM175 virus cDNA. cDNA clones were derived from pl6 HM175 virus RNA by cloning cDNA-RNA hybrid molecules into the PstI site of plasmid pBR322. The position and orientation of insert fragments within the HAV genome was determined by slot-blot hybridization with wild-type cDNA probes and by selected mapping of restriction endonuclease sites (See Report Number 1). Precise mapping of cDNA inserts was accomplished by dideoxynucleotide sequencing of plasmid DNA using pBR322 PstI site-specific oligonucleotide primers (Pharmacia). Insert fragments from pl6 cDNA clones were subcloned into the phage vector M13mp8 or M13mp9 and subjected to rapid sequencing using the dideoxynucleotide method of Sanger (Sanger et al., 1977). As physical mapping indicated few differences between pl6 virus cDNA and wild-type cDNA in terms of major restriction sites, a sequencing strategy was adopted which involved the subcloning and subsequent sequencing of overlapping restriction fragments. Where necessary, additional sequence information was obtained by direct sequence analysis of plasmid DNA using HAV-specific oligonucleotide primers prepared in the laboratory of C. Hutchison of the University of North Carolina or provided as a gift by J. Cohen of the National Institute of Allergy and Infectious Diseases, Bethesda, MD. The derived sequence for pl6 HAV cDNA was assembled with Micro-Genie software (Beckman) and compared with that reported by Cohen et al. for wild type (3rd marmoset passage) HM175 virus (Cohen et al., 1987a), and p35 chimpanzee-attenuated HM175 (Cohen et al., 1987b). Except where noted, mutations from the wild-type virus sequence were considered present only when found in at least two independent cDNA clones derived from pl6 virus.

The cDNA-RNA cloning strategy adopted permitted clones bearing stable HAV cDNA inserts to be obtained from minimal quantities of viral RNA. This approach was taken in order to limit the number of virus passages between plaque purification and isolation of viral RNA for molecular cloning. Clones derived from cDNA synthesized with oligo-dT₁₂₋₁₈ as primer were nearly always restricted to the 3' terminus of the genome, while clones of cDNA synthesized with random oligonucleotide primers were randomly distributed within the genome. Several clones containing 5' terminal sequences were identified, reflecting one advantage of the cloning of cDNA-RNA hybrid molecules (Cann et al., 1983; Stanway et al., 1983). Clones with overlapping inserts spanning the HAV genome were identified by hybridization, limited restriction mapping, and direct sequencing of plasmid DNA (Figure 1), and selected cDNA insert fragments were subcloned into M13 vectors for sequencing. The sequence of the complete genome was determined, with over 95% of the sequence confirmed in sequencing reactions involving both strands of cDNA.

Mutations in cell culture-adapted pl6 HM175. A total of 19 mutations (20 base changes) was evident when the sequence of pl6 HM175 was compared with that previously reported for wild-type HM175 virus (Cohen et al., 1987a) (Table 1). Except where indicated in Table 1, each of these mutations was

documented in at least two independent cDNA clones derived from pl6 virus. Mutations clustered in the 5' nontranslated region of the genome, and in the P2 and P3 regions (Figure 2). Altogether, there were 13 mutations in the large open reading frame, predicting 8 amino acid substitutions in the proteins of the pl6 virus (Table 1).

(i) 5' nontranslated RNA. There were five mutations (six nucleotide changes) in the 5' nontranslated RNA of the pl6 virus genome. At the 5' terminus, there was the addition of a U, confirmed in multiple cDNA clones, and between base positions 203-7 a UU deletion (map positions refer to published wild-type Hm175 sequence) (Cohen et al., 1987a). Thus, the length of the 5' nontranslated region was shortened by one base from the wild-type genome. The remaining changes included a G → A at position 8, an A → G at position 152 and a U → G at position 687.

(ii) P1 region. The capsid-encoding region was relatively free of mutations compared with other regions of the genome. One nucleotide substitution from the reported wild-type sequence was present in the putative VP3 coding region (position 1742), but did not predict a change in the amino acid sequence of the capsid protein. This silent "mutation" has been found in all cell-culture adapted Hm175 cDNA clones reported to date (Cohen et al., 1987b; Ross et al., 1986), and may in fact represent the true wild-type sequence, as the base in question has been determined in only a single wild-type cDNA clone (Cohen et al., 1987a). In addition, there was a mutation predicting a lysine to arginine substitution in amino acid residue 54 of VP2 (map position 964). This conservative substitution was the only predicted change in the capsid structure of the pl6 virion.

(iii) P2 region. There were six mutations in the P2 region, predicting four amino acid substitutions. According to the HAV polyprotein cleavage sites proposed by Cohen et al. (1987a), these changes included one amino acid substitution in protein 2A, two in protein 2B and one in protein 2C (Table 1).

(iv) P3 region. In the P3 region, there were 5 nucleotide substitutions predicting three amino acid substitutions. There were no predicted amino acid substitutions in proteins 3A or 3Cpro, the putative HAV protease. In the VPg (3B) protein (Weitz et al., 1986), however, there was a glutamine to histidine substitution at amino acid 11. Within the proposed polymerase coding region there were three mutations, two of which predicted amino acid substitutions in 3Dpol. These substitutions included a change from aspartic acid to glycine at residue 67 and from serine to threonine at residue 192.

(v) 3' non-translated RNA. A single mutation was present in the 3' nontranslated RNA, involving an A → G substitution at map position 7430.

Comparison of pl6 virus sequence with that of other cell-culture adapted Hm175 variants. The complete sequence of an Hm175 variant that had been adapted to growth in African green monkey kidney cells was recently reported by Cohen et al. (1987b). This p35 Hm175 variant was isolated directly from human feces and adapted to growth in vitro without prior marmoset passage. It has been shown to be attenuated in chimpanzees, and to a lesser degree in

marmosets (Karron, et al., 1988). In addition, the partial sequence of a third variant (p59) of HM175 virus has been reported by Ross et al. (1986). The p59 virus had been adapted to growth in monkey kidney cells following six marmoset passages, in vivo passages shared in common with the wild-type virus (first 3 marmoset passages) cloned by Ticehurst et al. (Cohen et al., 1987a) and the p16 virus (all 6 marmoset passages). Mutations common to these independently isolated viruses can be expected to have special relevance to the ability of virus to replicate in cell culture.

A comparison of the available sequence data shows that HM175 variants sequenced at the 16th, 35th and 59th passage levels have 6, 7, and 8 base substitutions, deletions or additions respectively, within the 5' nontranslated region of the viral genome. (The 5' terminal 29 bases have not been reported for the p59 virus.) The p16 and p35 sequences share a common mutation at base position 152 (A → G) and a common U deletion between 203-7. This latter change is also found in p59 virus, and is the only 5' mutation common to all three variants. The U → G substitution found at map position 687 in p16 virus is also present in the p59 variant, but is not found in p35 virus. Therefore, this mutation may have occurred during the last three marmoset passages which the p16 and p59 viruses shared in common. The region representing map positions 124 through 207 appears especially prone to develop mutations during in vitro passage of the virus (Jansen et al., 1987). Within this region, 3.6% of base residues were altered in p16 virus, as were 8.4% in the p35 sequence, and 2.4% in the p59 sequence. This mutational "hot spot" also contains two of the three 5' region mutations (map positions 152 and 203-7) common to two or more variants.

The full-length sequences of the p16 and p35 HM175 variants are remarkably concordant in terms of the distribution of mutations within the genome and share a striking number of identical or very similar mutations. In addition to the two identical mutations in the 5' nontranslated region, there are four mutations involving identical amino acids in the proteins of p16 and p35 viruses (Table 2). This represents 50% of the predicted amino acid substitutions present in p16 virus, and 33% of those present in p35 virus. In the P1 region, the single mutation (lysine → arginine) in VP2 of p16 virus is also present in p35 virus; a second mutation found in VP1 of p35 virus is not present in p16 HM175. In the P2 region, each variant has one mutation in protein 2A, and two in protein 2B (including a common alanine → valine substitution at residue 72). The greatest difference between the two viruses is in the 2C protein, in which there is one predicted amino acid substitution in p16 virus and four substitutions in the p35 variant. Neither virus has amino acid substitutions in proteins 3A or 3Cpro, but each virus has one substitution (at different sites) among the 23 amino acids of VPg (protein 3B). Of particular interest, there are two predicted amino acid substitutions in 3Dpol of both p16 and p35 viruses. These substitutions involve identical amino acid residues, including an identical substitution (serine → threonine) at residue 192, and different mutations involving aspartic acid 67. In p16, a mutation at map position 6148 predicts a substitution of this residue with glycine, while in p35 virus a mutation in the adjacent nucleotide (6147) predicts that this amino acid is replaced with asparagine. Lastly, a single, common mutation (A → G at position 7430) is present in the 3' non-translated region of both variants. With the possible exception of the apparent change at map

position 1742, these changes do not reflect errors in the reported sequence of wild-type HM175, as the wild-type sequence at the seven other sites of common mutations indicated in Table 2 was confirmed by Cohen et al. in at least two independent wild-type cDNA clones (Cohen et al., 1987a).

II. Construction of a genomic-length clone of p16 HM175 HAV cDNA

In an attempt to construct an infectious cDNA clone of virulent HAV, cDNA fragments from ten HM175 cDNA clones (Jansen et al., 1988; Ticehurst et al., 1983) have been used to assemble a genomic-length cDNA construct. This was accomplished by cleavage and ligation of overlapping inserts at convenient restriction enzyme sites (Figure 3). Wild-type HM175 cDNA (Ticehurst et al., 1983; Cohen et al., 1987a) containing sequence identical to the p16 consensus sequence was used in areas of the genome where no convenient restriction site was available in the p16 cDNA clones, or the sequence of individual p16 clones contained unique mutations not present in other clones derived from that region of the genome. Such mutations were not considered to be representative of the consensus p16 HM175 sequence. All ligation sites were subsequently sequenced to preclude the introduction of any sequence errors during the cloning procedure. The resulting full-length construct (pHAV_{ch}p16), cloned into the PstI site of pBR322, contained flanking dC-dG tails derived from the original cDNA-RNA cloning procedure (Jansen et al., 1988). This construct was transfected into BS-C-1 cells as supercoiled plasmid DNA, with calcium phosphate facilitation. The transfection control was pHAV/7 cDNA, derived from attenuated p35 HM175 virus (Cohen et al., 1987c) and obtained from R. Purcell of the NIAID. Transfected cell monolayers were grown under agarose overlays for two weeks at 36°C. The monolayers were then acetone fixed and stained with radiolabelled anti-HAV IgG and foci of virus replication detected by autoradiography (radioimmunofocus assay). HAV replication was detected with pHAV/7, but not pHAV_{ch}p16.

Cohen et al. (1987c) have suggested that homopolymeric dC-dG tails render genomic-length cDNA constructs of HAV non-infectious. We therefore removed the dC-dG tail at the 5' end of the construct using a polymerase chain reaction (PCR) based approach. Nucleotides 0-885 of the p16 genome were amplified from clone pHAV_{ch}119 (Figure 1) using a genomic-sense PCR primer from nucleotide 870-885 and an antigenomic-sense primer containing HAV specific sequence from nucleotides 0-14 with an additional seven nucleotides containing a HindIII restriction enzyme site immediately 5' of nucleotide 0 (note: nucleotide 0 is a terminally redundant U in the p16 genome, Jansen et al., 1988). The amplified product was subsequently cleaved with HindIII (nt 0) and XbaI (nt 745) and cloned into the pGEM3 transcription vector (Promega) between the HindIII and XbaI sites of the multiple cloning region, to create pHAV5'. A full-length construct of p16 lacking dC-dG tails at both the 5' and 3' ends was then assembled by ligation of fragments from pHAV5' (HAV nt 0-24), pHAV_{ch}p16 (nt 25-7003), and pHAV/7 (Cohen et al., 1987c) (nt 7004-7505) (Figure 4). The resulting construct (pHAV/p16) contains the consensus p16 HM175 sequence, minus a mutation at 7430 of the 3' noncoding region (Jansen et al., 1988), inserted between the HindIII and XbaI sites of the multiple cloning region of pGEM3 and without flanking dC-dG homopolymeric tails. pHAV/7 cDNA provided a convenient 3' end for the construct (nt 7004-7505) because it lacked a dC-dG tail and has a poly-A tail of sufficient length for

infectivity. cDNA in this region was originally derived from wild-type HM175 (Ticehurst et al., 1983) and differs from the pl6 sequence at only one base (nt 7430 of the 3' noncoding region) (Table 1). Absence of this nucleotide change has no demonstrable effect on HAV cDNA infectivity (Cohen et al., 1987c).

At the conclusion of the contract, the infectivity of this construct was under evaluation.

III. Characterization and partial nucleotide sequencing of cytopathic pHM175 HAV variants

pHM175 virus Persistent infection of BS-C-1 cells with pl6 HM175 virus was established as described previously (Cromeans et al., 1987). "pHM175" virus was recovered by disruption of these persistently infected cells after 21 to 23 subculture passages made at 2 to 4 week intervals. Unless otherwise noted, pHM175 virus stock had been serially passaged 4 times at low m.o.i. in FRhK-4 cells. By "serial passage", we mean the passage of virus recovered by disruption of infected cells. "43c" virus was twice plaque-purified from neutral red-stained overlays of FRhK-4 cells inoculated with pHM175 virus, and was selected for its ability to induce clearly visible plaques in this cell line. 43c stock virus was prepared in FRhK-4 cells. "18f" virus was twice clonally isolated from agarose overlying foci of viral replication in radioimmunofocus assays (Lemon and Jansen, 1985) (see below) of BS-C-1 cells inoculated with pHM175 virus; it was selected for continued reactivity with monoclonal antibody K24F2. 18f stock virus was prepared in BS-C-1 cells.

Antigenic variation among pHM175 variants Persistent HAV infection was established by inoculation of BS-C-1 cells with pl6 HM175 (Cromeans et al., 1987). Cells were subsequently subcultured at 2-4 week intervals. Rapidly replicating virus (pHM175) was recovered by disruption of cells after 21-23 subculture passages, and was shown to induce a cytopathic effect in both BS-C-1 cells and high-passage level FRhK-4 cells (Cromeans et al., 1987; Cromeans et al., 1989). We confirmed these results by establishing a plaque assay with HAV recovered by disruption of persistently infected BS-C-1 cells at the 21st-23rd passage level. Conventional plaque assays were carried out with either FRhK-4 or BS-C-1 cells. Following inoculation with pHM175 virus, cell sheets were overlaid with 0.5% agarose (Seakem ME, Rockland, ME) containing 2.0% heat-inactivated fetal calf serum and placed at 35 °C in a humidified environment with 5% CO₂. Six days later, a second overlay containing 0.5% agarose, 25mM MgCl₂ and neutral red (final concentration 0.02%) was added. Visible plaques were counted 6-24 hours later.

A clonal isolate (43c virus) was twice plaque-purified from the pHM175 virus harvest. When cell monolayers inoculated with 43c virus and overlaid with agarose were sequentially stained with neutral red and then processed for detection of radioimmunofoci, there was excellent concordance between the numbers of visible plaques and radioimmunofoci (not shown). Only occasional foci of viral replication that were visualized by staining with [¹²⁵I]-labeled polyclonal anti-HAV in the radioimmunofocus assay were not recognized as plaques following the staining of cell sheets with neutral red. Furthermore, autoradiograms of the [¹²⁵I]-anti-HAV stained cell sheets often

showed replication foci with central areas of cell necrosis, confirming the HAV-specific nature of the cytopathic effect.

As further proof that HAV was responsible for plaque formation, neutralization of the plaque-purified 43c virus was attempted with murine monoclonal antibodies to HAV in a plaque-reduction neutralization test. Surprisingly, these tests revealed that 43c virus was susceptible to neutralization with antibody K34C8 (83% plaque reduction at 0.1 mg/ml antibody), but resistant to antibody K24F2 (only 4.4% plaque reduction). These results were subsequently confirmed in a radioimmunofocus inhibition assay (not shown). The K34C8 and K24F2 monoclonal antibodies were raised against the HM790 strain of HAV (MacGregor et al., 1983), and both have been shown previously to neutralize HM175 virus following its adaptation to growth in cell culture (Stapleton and Lemon, 1987). These results thus indicated that passage of HM175 virus in persistently infected BS-C-1 cells and subsequent plaque selection of virus capable of inducing a cytopathic effect, all in the absence of applied antibody pressure, had resulted in isolation of virus with altered antigenicity and a K24F2 neutralization escape phenotype.

Relative replication advantages of K24F2-resistant and K24F2-sensitive virus during acute and persistent infections We examined several different passage levels of the persistently infected BS-C-1 cells, and harvests made from subsequent serial passage of pHM175 virus in BS-C-1 and FRhK-4 cells. The epitope-specific radioimmunofocus assay employed in these studies involved sequential staining of infected cell sheets with radioiodinated monoclonal (K24F2) and polyclonal (JC) antibodies. Replication foci of virus bearing the K24F2 epitope (K24F2⁺, wild-type antigenic phenotype) were visualized by autoradiography following reaction with either the monoclonal antibody or the polyclonal antibody, while foci of K24F2 resistant mutants (K24F2⁻ phenotype) were recognized only by staining with polyclonal antibody. This approach revealed that 99% of the infectious virus particles present in disrupted cell lysates from the 23rd passage of persistently infected BS-C-1 cells were of the mutant K24F2⁻ phenotype. However, with subsequent serial passage of this virus in FRhK-4 cells, virus with a normal antigenic phenotype (K24F2⁺) was selected over K24F2⁻ virus. The K24F2⁺ virus represented 92-97% of all infectious virus after 3-4 serial passages in FRhK-4 cells (see Report Number 3 for specific details of these studies).

Both K24F2-resistant and K24F2-sensitive pHM175 virus induce a cytopathic effect It was of interest to determine whether K24F2⁺ pHM175 virus was also capable of inducing a cytopathic effect. Accordingly, a K24F2⁺ virus variant (18f virus) was clonally isolated from the agarose overlay of an epitope-specific radioimmunofocus assay carried out in BS-C-1 cells. Unlike the 43c virus which was selected for its ability to induce distinct plaques, 18f virus was selected for its continuing ability to react with K24F2 antibody. 18f virus was shown to have a rapidly replicating phenotype, as it formed large radioimmunofoci within 7 days after inoculation of cells. Similar size replication foci require approximately 14 days incubation with the parental pl6 virus. Furthermore, 18f virus produced cytopathic effects in both BS-C-1 and FRhK-4 cells matching those found with the K24F2⁻ 43c virus (Table 3). These cytopathic changes were similar to those described previously (Anderson, 1987a; Cromeans et al., 1987), and included the

rounding-up of cells followed by their release from the cell sheet. 18f virus generated distinct plaques in BS-C-1 cells maintained under agarose and stained with neutral red (not shown). The growth properties of 18f and 43c viruses were similar (Table 3). These studies thus demonstrated that escape from neutralization by K24F2 was neither necessary nor specifically associated with the ability of HM175 virus variants to generate a cytopathic effect in cell culture.

Capsid mutations associated with K24F2 neutralization escape, persistent infection, and cytopathic phenotype To determine capsid protein mutations in 43c and 18f viruses, we sequenced the P1 regions of genomic RNA extracted from these variants and compared these sequences with those of wild-type (3 marmoset passages) (Cohen et al., 1987a) and pl6 HM175 virus (Jansen et al., 1988) (Table 4). The change at residue 2-054 which was found in the parent pl6 virus was also present in both 43c and 18f viruses, as was the single silent mutation at base 1742. However, in the K24F2⁺ 18f virus, additional mutations were identified by RNA sequencing at residues 3-091 (threonine to lysine), and 1-271 (serine to proline) (Table 4). Interestingly, the substitution at residue 3-091 in 18f virus occurred as a result of a mutation in the base (1741) adjoining the silent mutation found in all the cell culture-adapted variants, resulting in a change in two of the bases present in the wild-type codon. As previously reported (Ping et al., 1988), mutations were found at residues 3-070 (aspartic acid to alanine), 1-197 (asparagine to serine) and 1-276 (methionine to valine) of the K24F2⁻ 43c virus. Thus, remarkably similar mutations were found in the 43c and 18f cytopathic viruses, with mutations present in both viruses in regions bounded by residues 70 to 91 of VP3, and 271 to 276 of VP1. Outside these regions, only two additional mutations (from the noncytopathic pl6 virus) were noted in the P1 genomic sequences of 43c and 18f viruses, one of which was silent (base 2684 of 18f). These data thus indicate a relatively low rate of mutation in the P1 region of the genome during persistent infection over the course of almost one year.

Comparative antigenicity of cytopathic 43c and 18f HM175 variants We carried out a detailed comparison of the antigenicity of 43c, 18f and pl6 viruses, using a panel of neutralizing murine monoclonal antibodies assembled from several laboratories. Standardized quantities of each virus (determined by cDNA-RNA hybridization) were tested for ability to bind monoclonal antibodies in an indirect radioimmunoassay (Ping et al., 1988). While these assays demonstrated that 43c virus was poorly recognized by many of the monoclonal antibodies, the 18f variant was indistinguishable from the noncytopathic pl6 virus (for details, see Report Number 3). As further evidence of the antigenic differences between these viruses, standardized quantities of each virus were tested in a polyclonal radioimmunoassay. These results indicated that the few capsid mutations present in 43c virus resulted in a marked reduction in the antigenicity of the virus that was detectable in a polyclonal antibody-based assay. This finding supports the concept that the HAV capsid displays an immunodominant antigenic site on its surface (Stapleton and Lemon, 1987; Ping et al., 1988). In contrast, the mutations present in the 18f capsid did not result in demonstrable changes in antigenicity in either monoclonal or polyclonal radioimmunoassays. These findings have been confirmed in subsequent monoclonal antibody neutralization assays.

Thermal stability of cytopathic HM175 variants Since it has been suggested that another cytopathic variant arising from HM175 virus during persistent infection in vitro might have reduced virion stability at elevated temperatures (Anderson, 1987), we determined whether the mutations present in 43c and 18f variants affected the thermal stability of these viruses. Crude cell lysates containing pl6, 43c and 18f viruses were heated for 10 min at temperatures ranging from 40 °C to 90 °C, then assayed for infectious virus at 35 °C (for details, see Report Number 3), and for HAV antigen detectable in a polyclonal solid-phase radioimmunoassay. Incubations were carried out in the absence or presence of additional Mg^{++} (1 M $MgCl_2$), as this divalent cation is known to stabilize the HAV capsid against heat denaturation (Siegl et al., 1984). These studies failed to demonstrate reproducible differences between the thermal stability of the pl6 virus and that of the cytopathic 43c and 18f variants, indicating that the rapidly replicating cytopathic phenotype was not associated with reduced thermal stability of the virus capsid. With increasing temperatures, significant decrements in infectivity of each virus were noted before comparable reductions in antigenicity, suggesting heat-related conversion of virus to noninfectious particles that retained antigenicity. As expected, the addition of 1 M Mg^{++} resulted in an increase of approximately 20 °C in the 50% survival point of all three viruses.

IV. Virulence of a neutralization escape mutant derived from pl6 HM175

HM175/S18 virus HM175/S18, a neutralization resistant variant of HM175 virus was isolated by repetitive cycles of neutralization with monoclonal antibody K24F2, followed by amplification and plaque-purification as described previously (Stapleton, 1987). This virus escapes neutralization by many anti-HAV monoclonal antibodies, due to a replacement of the aspartic acid 70 residue of the capsid protein VP3 with histidine (Ping et al., 1988).

Animal challenge experiments Experimental infection of healthy, seronegative New World owl monkeys (*Aotus trivirgatus*) was carried out as described previously (LeDuc, 1983), in collaboration with COL J. LeDuc, Ph.D. (USAMRIID) and L. Binn, Ph.D. (WRAIR). Six seronegative owl monkeys were inoculated intravenously with 5.3×10^4 RFU of pl HM175/S18 virus. All six monkeys seroconverted to anti-HAV positive in the HAVAB radioimmunoassay by 32 days postinoculation. Elevations of serum ALT activities were noted in all six monkeys, as was fecal shedding of HAV antigen detectable by solid-phase radioimmunoassay (for details of these studies, see Report Number 2). The response to intravenous inoculation of HM175/S18 virus was compared to that following intravenous inoculation of wild-type HM175 virus (human fecal suspension), given to five owl monkeys in a previous study (LeDuc et al., 1983). The mean maximum fold-increase in serum ALT in monkeys infected with HM175/S18 was 6.5 (range 3.8-11.3), compared with 8.5 (range 2.6-16.5) in monkeys infected with wild-type virus. The period from inoculation of HM175/S18 to maximal ALT elevation was 28.2 days (range 22-33), compared with 34.8 days (range 25-43) for wild-type virus. Antigen shedding in feces appeared to be reduced in HM175/S18 virus-infected monkeys (mean maximum S/N 16.2, range 3.4-36.4) compared with monkeys infected with wild-type virus (mean maximum S/N 73.2, range 24-112).

Because HM175/S18 virus is highly adapted to growth in cell culture, it was possible to efficiently recover virus from fecal suspensions and serum specimens collected from infected owl monkeys (Lemon et al., 1989) (Figure 5). In three monkeys, virus was first noted at low titers in fecal suspensions 4 days after intravenous inoculation and was present in the feces of all monkeys by day 6. Epitope-specific radioimmunofocus assays demonstrated that virus shed in the first few days of infection was of the mutant phenotype, but that such virus was replaced by day 10 with virus which was antigenically normal (i.e., reactive with the monoclonal K24F2) (Lemon et al., 1989). Fecal virus shedding peaked in all monkeys prior to maximum elevations of serum amino-transferase activities, but sporadic shedding of small quantities of virus occurred as late as 35-42 days after inoculation (Figure 5). Fecal virus shedding was not noted in subsequent specimens collected between 75 and 102 days. Viremia was documented in all six monkeys inoculated with HM175/S18 virus. The course of the viremia generally paralleled fecal shedding of virus, but the quantity of virus present in serum was always several orders of magnitude less than that in feces. Neutralizing antibody was present in each of the owl monkeys by 35 days after inoculation, and by 21 days in animals I2, A5, and PN2.

Neutralization phenotype of HM175/S18 virus after monkey passage. Virus present in fecal specimens collected 21 days postinoculation from both monkeys was tested in a log-reduction neutralization assay, against monoclonal K24F2 and compared with pl HM175/S18 virus and its pl5 HM175 parent. Virus from PN2 and J1 demonstrated a neutralization susceptibility (1.16 and 1.12 log₁₀ reduction) approaching that of the pl5 HM175 parent virus (1.57 and 1.36 log₁₀ reduction), confirming the epitope-specific radioimmunofocus assays described above. These findings were further confirmed by direct sequencing of RNA from virus recovered from animal PN2, which demonstrated reversion to the wild-type aspartic acid residue at position 70 of VP3 (Lemon et al., 1989). Thus, passage of HM175/S18 in the owl monkey was associated with selection of revertant virus.

V. Development of an antigen-capture/polymerase chain reaction (AC/PCR) method for strain-specific identification of HAV in human fecal materials

Sensitivity of AC/PCR The method was described in detail in Report Number 3 of this contract. Only results will be summarized here. To evaluate the sensitivity of AC/PCR, we compared its ability to detect viral nucleic acid with a conventional PCR method involving the enzymatic amplification of cDNA made from HAV RNA that had been prepared by phenol/chloroform extraction following incubation of virus with SDS and proteinase K. Serial dilutions of a suspension of feces collected from an experimentally infected owl monkey were tested in parallel by both methods. A region in the viral genome 206 nucleotides in length and encoding capsid protein VP3 near its carboxy terminus was selected for amplification by PCR. In replicate experiments, both AC/PCR and the conventional PCR method yielded a single amplified reaction product of the expected length, confirmed to contain the appropriate HAV sequence by Southern hybridization. The end-point dilutions were similar for both methods, indicating that heat-denatured, affinity-isolated virus provided template of sufficient purity for subsequent polymerase reactions.

Since the fecal specimen tested in this experiment was collected from an owl monkey that had been inoculated with a cell culture-adapted HAV variant (see above; Lemon et al., 1989), we were able to compare the detection limits of AC/PCR with the infectious titer determined by an in vitro radioimmunofocus assay ($10^{7.3}$ radioimmunofocus-forming units (RFU)/g of the original fecal sample). With serial dilutions of the fecal suspension down to that containing as little as 0.47 RFU in the tested sample, AC/PCR resulted in progressively lesser amounts of the amplified reaction product. A weak signal was detected at 0.047 RFU, while an isolated positive result was obtained at 0.0047 RFU. PCR amplification of cDNA derived from phenol/chloroform-extracted viral RNA appeared similarly quantitative with respect to input virus, with reaction products consistently detected down to 0.047 RFU (see Report Number 3). Previous experiments in our laboratory have suggested that the particle/infectivity ratio of cell culture-adapted HAV is approximately 60:1 (see Report Number 2; Jansen et al., 1988); thus, the limits of detection by both techniques is in the range of 3 to 30 virus particles.

Specificity of AC/PCR HAV-containing samples were placed into reaction tubes coated with monoclonal antibodies to HAV, poliovirus type 1 (PV1), or respiratory syncytial virus (RSV) for assay by AC/PCR. Strong hybridization signals were obtained with products from reaction tubes coated with anti-HAV monoclonal antibody, while residual virus present in reaction vessels coated with nonspecific antibodies led only occasionally to low levels of amplified HAV reaction products detectable by Southern hybridization.

Application of AC/PCR: Comparison with Conventional Methods for Detection of HAV Fecal specimens from 21 acutely infected American soldiers were examined for the presence of HAV by means of AC/PCR. These specimens had been collected during the first week of illness from IgM anti-HAV positive soldiers involved in a common source outbreak of hepatitis A which occurred during a field exercise at Grafenwohr, in the Federal Republic of Germany, during the summer of 1982 (see relevant EPICON report; Lednar et al., 1985). Seventeen of 21 (81%) individual fecal specimens yielded reaction products (206 bp) that were visualized in an ethidium bromide-stained agarose gel. Further analysis by Southern hybridization revealed 2 additional positive samples (data not shown), bringing the total number of HAV-positive specimens to 19 (90%). The sensitivity of AC/PCR was thus significantly greater than solid-phase radioimmunoassay and immunoaffinity cDNA/RNA hybridization (Jansen et al., 1985), two methods commonly used for detection of HAV in clinical samples. Of 18 fecal specimens that were positive for HAV by AC/PCR, only 13 were positive by cDNA-RNA hybridization. Ten of these specimens contained HAV antigen detectable by solid-phase radioimmunoassay. In general, there was a quantitative relationship between the cDNA-RNA hybridization blot intensity, radioimmunoassay sample/negative control (S/N) ratio, and the quantity of PCR-amplified product obtained from each positive fecal sample (not shown). These results suggest that AC/PCR is at least semi-quantitative under the conditions employed.

Molecular Epidemiology of HAV Reaction products amplified by AC/PCR from clinical samples (or cell culture materials) containing HAV obtained in different epidemiologic settings were sequenced by extension of labelled primers in the presence of dideoxynucleotides. To compare HAV strains, we

analyzed colinear sequences of each virus derived from two different genomic regions. These regions spanned nucleotides 2056 to 2208 (carboxy terminal region of VP3), and 3020 to 3191 (VP1/2A junction region) in the viral genome. The 325 nucleotide bases thus included in this analysis represent approximately 5% of the viral genome. We found no differences in the sequence of AC/PCR reaction products derived from cell culture-adapted HM175, CR326 or HAS15 strains of HAV, and the sequences of these viruses reported previously from molecularly cloned cDNA (Cohen et al., 1987a; Linemeyer et al., 1985; Ovchinnikov et al., 1985), demonstrating the accuracy of sequence data derived from viral RNA by AC/PCR. The AC/PCR-derived sequence of strain MBB fecal material differed from the reported cDNA-derived sequence at a single base position (Paul et al., 1987). However, substantial differences (up to 9.8% nucleotide nonidentity) were found between AC/PCR reaction products of HAV strains obtained in different epidemiologic settings. These results allowed construction of a dendrogram showing genetic relatedness among these strains (Figure 6). Similar results were obtained by independent comparisons within each genomic region (VP3 and VP1/2A), indicating that the sample size was sufficient for reliable measures of relatedness.

Viruses present in fecal samples collected from three soldiers (GR-1, GR-7 and GR-CL) involved in the 1982 Grafenwohr outbreak of hepatitis A in the Federal Republic of Germany shared a common nucleotide sequence, as might be expected. Similarly, fecal specimens collected from three cases of endemic hepatitis A occurring in central Greece over a 5 month period during 1983 (AG11, AG5978, and AG6014) contained viruses that were identical or differed at a single base position, suggesting the presence of a single virus circulating in Greece at that time. However, the sequences of the viruses recovered in Greece and in Germany differed from each other by 9.8%. As this difference is as great as that between any two strains of human HAV, these data indicate the existence of distinct strains of HAV in Northern and Southern Europe during 1982-1983 and suggest a new view of the epidemiology of HAV in human populations.

Of particular interest was the fact that fecal specimen LV-BE contained virus with a sequence identical to the virus causing the German epidemic. LV-BE was collected from an American soldier involved in an epidemic of hepatitis A which occurred at the military prison at Fort Leavenworth, Kansas during the summer of 1982 (see relevant EPICON report; Lednar et al., 1985). The fact that the sequence of this virus was identical to that causing a simultaneous outbreak among American soldiers in Europe establishes a clear but previously unrecognized epidemiologic link between these two HAV epidemics. This result dramatically underscores the power of this approach to studying the epidemiology of HAV at the molecular level.

Also surprising was the fact that HAS-15 and LCDC viruses (obtained from the Laboratory for the Center for Disease Control, Ottawa, Canada) shared identical sequences. This result may indicate very close relatedness two among diverse isolates (HAS-15 was recovered near Phoenix, AZ, while LCDC was recovered human feces collected from an ill patient in China). However, several other cell culture derived "isolates" from other laboratories appear to be contaminants derived from prototype laboratory strains and not original isolations of HAV. We have compared the nucleotide sequence of such

"isolates" with that of virus in fecal material. An example is the KMW-1 strain of HAV: the sequence of this virus when studied from feces is markedly different from that of the related cell culture-adapted isolate (both obtained from G. Siegl, Bern, Switzerland), which is itself identical to the MBB strain. In this case it is certain that the KMW-1 "isolate" is an MBB contaminant, as MBB virus was also under culture in this laboratory. Since the LCDC sequence was derived from virus propagated in cell culture, a firm conclusion concerning the relatedness of LCDC and HAS-15 must await the testing of LCDC virus present in fecal samples.

VI. Partial genomic sequence of the PA21 strain of HAV recovered from New World Panamanian owl monkeys.

RNA was isolated from gradient-purified PA21 virus. Random oligonucleotide primers were used to generate double-stranded cDNA by the method of Gubler and Hoffman (Brown et al., 1989). The cDNA was blunt-ended with T4 DNA polymerase and ligated to EcoRI linkers using T4 DNA ligase, followed by digestion with EcoRI. The cDNA was ligated into the EcoRI site of the plasmid vector pTZ18R (U.S. Biochemical) and the cDNA-vector hybrids were used to transform competent *E. coli* NM522 cells. Ampicillin resistant clones were screened by in situ colony hybridization using [³²P]-labeled, randomly primed cDNA prepared from PA21 RNA. Single-stranded DNA was prepared from positive clones and sequenced using the dideoxy chain termination method of Sanger.

Overlapping clones spanning part of the 5' noncoding region, the complete P1 region and the 5' portion of the P2 region of PA21 were sequenced completely, and the derived sequence compared with known sequences of human HAV strains. Additional sequence was obtained from 3' noncoding and the 3' terminus of the P3 genomic region. The 5' noncoding, P2, P3 and 3' noncoding regions were found to have 89.2%, 79.6%, 84.4%, and 93.7% nucleotide identity with respect to the human HM175 virus (Brown et al., 1989). The G+C content of PA21 RNA was found to be 39.5%, only slightly higher than that of other HAV strains. The codon usage was unremarkable except for the leucine TTA codon which is used by PA21 with less than half the frequency of the human HAV strains (5/63 vs 13/63 for HM175). The typical HAV bias against the CG nucleotide pair was present (frequency of use 0.4%).

The P1 region of PA21 genomic RNA was found to comprise 2373 nucleotides and to encode 791 amino acids. The nucleotide difference between PA21 and sequenced human HAV strains was approximately 83% in the capsid-encoding region. The region encoding VP1 (Figure 7) demonstrated the greatest divergence (78.4% - 81.3% identity with 6 human HAV strains), while the VP3 encoding region, as in comparisons of other picornaviruses, was the most conserved (84.7% - 86.0% identity). The majority of the nucleotide changes occur in the third codon position (343 of 398, in comparison with HM175), while differences present in the first or second positions often do not lead to a change in the amino acid. Thus, the amino acid sequence of the capsid proteins is largely conserved between the simian and human viruses.

Overall, the PA21 P1 genomic region sequence indicates that its capsid differs at only 23 (HM175 and MBB) to 31 (HAS15) amino acid residues when compared with the capsids of human HAV strains. Most of these changes (16 out of 23) are conservative in nature. Although the nucleotide changes are distributed randomly across the P1 region (except for the region encoding the carboxy terminus of VP1, at the 3' end of the P1 region), amino acid replacements are clustered near the amino termini of VP2 and VP1, and the carboxyl terminus of VP1 (for details, see Brown et al., 1989). Of the 31 carboxy terminal amino acids of VP1, eight are different from HM175 virus.

CONCLUSIONS

Mechanisms of cell culture-adaptation and attenuation of HAV. Because adaptation of HAV to growth in cell culture has been the first step in the development of all attenuated variants examined to date (Provost et al., 1982; Provost et al., 1986; Karron, et al., 1988), an understanding of the molecular basis of attenuation of HAV is dependent on understanding the events accompanying adaptation of the virus to cell culture. The mutations identified in the pl6 variant of HM175 virus provide information that is helpful in understanding the molecular basis of cell culture adaptation.

The pl6 variant is highly adapted to growth in vitro. However, experimental infection of New World owl monkeys with a neutralization-resistant variant of HM175 virus that is closely related by passage history to pl6 virus demonstrated nearly unaltered virulence at the 22nd in vitro passage level (Lemon et al., 1987a; Lemon et al., 1989). This suggests that the mutations present in pl6 virus have not resulted in significant attenuation for owl monkeys. Thus, comparison of the pl6 virus (cell culture adapted, but virulent) sequence with that of the p35 virus (cell culture adapted, attenuated) (Cohen et al., 1987b) provides useful insights into the possible molecular basis of attenuation of HAV as well as cell culture adaptation.

The lack of mutations predicting changes in the surface structures of the pl6 virus argues against alterations in virus receptor-binding activity as a mechanism of adaptation to growth in vitro. The mutation in VP2 that is common to both pl6 and p35 variants appears unlikely to have induced changes in the receptor-binding activity of the virus, given that the position of this residue is probably deep within the capsid structure according to computer-generated alignments derived by A. Palmenberg (personal communication) and the known crystal structure of other picornaviruses (Luo et al., 1987; M. Rossmann, personal communication). However, the precise location of this VP2 residue in the HAV capsid and the possible functional relevance of this mutation must await crystallographic determination of the HAV capsid structure. A second reason for believing the change in VP2 is not essential for adaptation of virus to growth in cell culture is that this mutation is not present in the cell culture-adapted p59 variant of HM175 (Ross et al., 1986). Similarly, the absence of mutations in the putative protease, 3Cpro, or at proposed cleavage sites for polyprotein processing in HAV (Cohen et al., 1987a), suggests that changes in posttranslational processing do not play major roles in adaptation of virus to cell culture.

Mutations within the 5' and 3' nontranslated regions of the genomes of pl6 and p35 viruses could affect priming or initiation of both positive and negative strand RNA replication. Since guanidine resistance of poliovirus maps to the 2C protein of that virus (Pincus et al., 1986), mutations present in the analogous HAV protein may also affect viral RNA replication. The coevolution of related mutations in the polymerase (3Dpol) proteins of both pl6 and p35 viruses (Table 2) argues that changes in polymerase function and hence RNA replication may be central to efficient growth in vitro. Such mutations could result in more efficient interactions between viral proteins, viral RNA and cell-specific proteins suspected to be involved in replication of picornaviral RNA (Andrews et al., 1986; Morrow et al., 1985). This

interpretation is consistent with evidence suggesting that the restrictive event in HAV replication in vitro may be replication of the viral RNA (Anderson et al., 1987).

Host cell factors necessary for replication of viral RNA in cultured monkey kidney cells may be different from those available to the virus in the human hepatocyte, the cell type within which virus replication normally occurs in vivo. If so, mutations occurring during adaptation of virus to growth in cell culture and permitting more efficient utilization of host cell factors in vitro might be expected to lead to a reduction in the ability of virus to replicate within the hepatocyte in vivo. This hypothesis provides a possible explanation for the attenuation of HAV that has been associated with passage of the virus in cell cultures. It is consistent with the observation that attenuated HAV (specifically, the F' variant of CR326 strain HAV) replicate only to low levels in nonimmune humans, as evidenced by delayed and relatively low level antibody responses (Provost et al., 1986; S.M. Lemon and P. Provost, unpublished data). Attenuation of HAV, like attenuation of poliovirus (Sabin, 1985), may thus be related to mutations in several regions of the genome (Minor et al., 1986; Omata et al., 1986).

An effect on ribosome recognition or initiation of translation cannot be ruled out for the nucleotide changes found in the 5' nontranslated region of the genomes of cell culture-adapted HAV variants. The functions of the nontranslated 5' RNA leader sequence of picornaviruses remain poorly defined, although substantial progress has been made in this area in recent years. Specific ribosomal binding domains (ribosomal landing pads) have been identified within the 5' noncoding region of some picornaviruses (e.g., poliovirus), and there is now a general understanding that translation of noncapped picornaviral messenger RNA occurs as a result of internal binding of the ribosome with only limited ribosomal scanning (Pelletier and Sonenberg, 1988). Complementary data for HAV are not yet available, however, and thus it is not possible at present to relate the mutations identified in the 5' noncoding region of p16 HM175 virus to this specific function. Nor can the influence of mutations on viral assembly, or on the efficiency of virus uncoating following penetration be ascertained. The recent availability of an infectious cDNA construct (pHAV/7) derived from the p35 variant of HM175 (Cohen et al., 1987c) will, however, allow more precise mapping of viral functions involved in adaptation of virus to in vitro growth and may possibly shed light on their relevance to attenuation.

Genomic-length p16 HM175 virus cDNA construct The infectious construct developed by Cohen et al., (1987c), pHAV/7 was derived from highly attenuated virus (p35 HM175). For several reasons, an infectious construct derived from a virulent virus (such as p16 HM175) would be of benefit in further studies of the molecular basis of attenuation. The p16 HM175 cDNA clones described above have thus been assembled into a genomic length construct. Flanking dC-dG tails remaining from the original homopolymeric RNA/cDNA cloning procedure were removed, and the full-length p16 sequence (minus one mutation in the 3' noncoding region which is nonessential for infectivity in vitro, Cohen et al., 1987c) has been inserted into the vector pGEM3 immediately downstream of the SP6 transcriptional start site. Studies defining the infectivity of this plasmid (either as DNA or RNA) remain in progress, but it is expected that

this genomic length construct will be of considerable value in further defining the critical mutations associated with adaptation of p16 HM175 to growth in cell culture. Furthermore, this construct may provide the means of attempting alternate approaches to attenuation of the virus. Deletion of regions of the 5' noncoding region of wild-type poliovirus has been shown to result in substantial attenuation (Nomoto et al., 1987). Such attenuated mutants should be extremely stable with little chance of reversion to virulence.

Cytopathic variants of HM175 strain HAV Currently there is very little understanding of the mechanism underlying the cytopathic effect observed with some cell culture-adapted variants of HAV. In general, these cytopathic HAVs appear to be capable of relatively rapid, efficient replication in cell culture when compared with noncytopathic but otherwise cell culture-adapted HAV variants (Table 3) (Anderson, 1987; Cromeans et al., 1987; Nasser and Metcalf, 1987). Cytopathic HAV variants do not appear to interfere with host cell macromolecular synthesis. It is likely that multiple mutations play a role in determining the cytopathic phenotype, just as multiple mutations appear to be important in the primary adaptation of virus to growth in vitro (Jansen et al., 1988; Cohen et al., 1987b). From a practical point of view, the in vitro growth characteristics of some of these cytopathic variants (e.g., the 18f variant) may make them excellent candidates for development of inactivated vaccines or the production of viral antigen for diagnostic tests.

We defined capsid mutations present in two rapidly replicating, cytopathic HM175 virus variants. We demonstrated that an antigenically altered K24F2⁻ virus variant was spontaneously selected during passage of cells that were persistently infected with HM175 virus (in the absence of antibody pressure). This variant (43c virus) grew relatively rapidly and was cytopathic in both FRhk-4 and BS-C-1 cells; it had amino acid substitutions at residues 3-070 (i.e., residue 70 of VP3), 1-197, and 1-276. The mutation at 3-070 is most likely responsible for antigenic variation in 43c virus, as mutation at this residue has been associated with neutralization escape from K24F2 in viruses specifically selected for resistance to antibody (Ping et al., 1988). However, a role for the other capsid mutations in the altered antigenicity of 43c virus (particularly the mutation at 1-276) has yet to be excluded. A second cytopathic HM175 variant was isolated from the same persistently infected cell cultures; this variant (18f virus) had mutations at similar sites in the capsid proteins (residues 3-091 and 1-271) but was antigenically indistinguishable from the parent HM175 virus in solid-phase immunoassays and virus neutralization tests with monoclonal antibodies.

Several explanations are possible for the emergence of an antigenically altered virus during persistent HAV infection. Mutations in the region of VP3 residues 3-070 to 3-091 and VP1 residues 1-271 to 1-276 may influence replication of the virus during persistent infection and serial passage in vitro, as they were present in both the 43c and 18f cytopathic variants, but the extent to which they do so remains uncertain. It is likely that mutations elsewhere in the genome play important roles in determining the cytopathic phenotype. Mutations in the 5' noncoding region and in the P2 (proteins 2B and 2C) and P3 (3D_{pol}) genomic regions appear to be important in the initial adaptation of virus to growth in cell culture (Cohen et al., 1987b; Jansen et

al., 1988), and thus may also play dominant roles in the evolution of virus to a rapidly replicating cytopathic phenotype. In support of this hypothesis, we have recently determined that 43c and 18f viruses share unique mutations at bases 591 and 647 within the 5' noncoding region. Further sequencing efforts and the construction of infectious cDNA from cytopathic HAV variants will be necessary to resolve this issue.

Neutralization resistance of HAV and hepatovirulence. Previous studies with neutralization-escape mutants of other RNA viruses, including some picornaviruses (Prabhakar, 1987), have demonstrated reduced virulence in comparison with wild-type virus. We found, however, that the escape mutant HM175/S18 (which contains an aspartic acid to histidine replacement at residue 3-070) remains hepatovirulent in owl monkeys. Although fecal shedding was apparently reduced in comparison with animals infected previously with a wild-type HM175 inoculum (Lemon et al., 1987a), the incubation period and magnitude of liver enzyme elevations were similar in monkeys infected with wild-type and neutralization-resistant HM175 virus. Virus shed in the feces of HM175/S18 infected monkeys during the first 10 days of infection maintained the neutralization escape phenotype (Lemon et al., 1989), but virus shed beyond 10 days had reverted to a neutralization susceptible phenotype and had regained the wild-type sequence. It is likely that hepatic disease in these owl monkeys was related to replication of the revertant virus that was selected for in each of the inoculated animals.

The reversion of virus to a neutralization-susceptible phenotype in each of these monkeys supports the hypothesis that the major antigenic domains of HAV are highly conserved because they are essential for efficient replication in vivo, but leaves the intrinsic pathogenicity of neutralization escape variants of HAV unresolved. This could be assessed by animal challenge with a stable HM175 escape mutant containing an aspartic acid to serine mutation at residue 3-070, recently generated by site-directed mutagenesis of the infectious pHAV/7 clone in collaborative experiments between our laboratory and Stephen Feinstone, M.D., of the Hepatitis Section, NIAID (Cox et al., in preparation). The mutation in this synthetic virus was created by inducing changes at two consecutive nucleotides and should be relatively resistant to reversion compared with the single nucleotide substitution present in HM175/S18 (Ping et al., 1988).

AC/PCR and limited sequence analysis of wild-type viruses With the testing of new HAV vaccines by the U.S. Army and other agencies, there is a growing need for a rapid and sensitive method capable of detecting and distinguishing specific strains of HAV in human samples. Our results demonstrate the utility of AC/PCR as a diagnostic procedure and support its use in characterizing the molecular epidemiology of viruses such as HAV. It is applicable to the testing of substantial numbers of specimens in epidemiologic or clinical studies, including future HAV vaccine trials.

Rico-Hesse and coworkers (Rico-Hesse et al., 1987) have demonstrated that contemporary isolates of single poliovirus serotypes commonly share less than 85% nucleotide identity. We found considerably greater genetic relatedness among human HAV isolates collected worldwide (Figure 6), although HAV and polioviruses share many other common genomic features. Remarkably,

the MS-1 and NC-1 strains of HAV (collected 25 years apart) have nucleotide sequences that are 96.6% identical and show no amino acid changes within the regions sequenced. While conservation of the primary structure of HAV capsid proteins may reflect structural constraints imposed on the evolutionary process, considerable divergence in the amino acid sequence of the carboxy terminus of VP1 has been found recently in the PA21 simian strain of HAV. Thus there must be other reasons for the high level of conservation that we found among human HAV strains.

Molecular cloning and partial nucleotide sequencing of PA21 virus

Cloning and sequencing studies indicate that PA21 virus represents a unique genotype of HAV and suggest the existence of an ecologically isolated niche for HAV among feral owl monkeys.

Table 1. Comparison of wild-type and cell culture-adapted
p16 HM175 virus

		Nucleotide			Amino acid		
		wt					
Region		pos	wt	p16	pos	wt	p16
5'		0	-	U			
		8	G	A			
		152	A	G			
		203-7	UU	dd			
		687	U	G			
P1	VP2	964	A	G	54	lys	arg
	VP3	1742	G	A*			
P2	2A	3281	A	G	58	ile	met
	2B	3711	G	A	13	asp	asn
	2B	3867	U	C*			
	2B	3889	C	U*	72	ala	val
	2C	4049	C	U*			
	2C	4426	A	C	144	lys	thr
P3	3A	5204	G	A			
	VPg	5255	A	U	11	gln	his
	3Dpol	6148	A	G	67	asp	gly
	3Dpol	6216	U	C			
	3Dpol	6522	U	A	192	ser	thr
3'		7430	A	G			

* Identified in only one clone from p16 virus;
all other mutations confirmed in at least
two clones. The mutation at 1742 has been
confirmed by direct sequencing of viral RNA
(unpublished data).

d - Deletion

Table 2. Mutations in independently isolated
cell culture-adapted HML75 variants

Region		nucleotide or amino acid position*	wt	p16	p35	
5'		0	-	U		
		8	G	A		
		124	U		C	
		131-134	UUUG		dddd	
		152	A	G	G	†
		203-207	UU	dd	dU	†
		687	U	G		
P1	VP2	54	lys	arg	arg	†
	VP1	273	glu		val	
P2	2A	30	asn		ser	
	2A	58	ile	met		
	2B	13	asp	asn		
	2B	72	ala	val	val	†
	2B	82	gly		ala	
	2C	29	lys		met	
	2C	64	glu		lys	
	2C	76	phe		ser	
	2C	144	lys	thr		
	2C	190	val		ile	
P3	VPg	4	his		tyr	
	VPg	11	gln	his		
	3Dpol	67	asp	gly	asn	†
	3Dpol	192	ser	thr	thr	†
3'		7430	A	G	G	†

* Based on polyprotein cleavage sites proposed by Cohen et al. (1987a).

† Similar or identical mutations (only mutations in 5' and 3' nontranslated RNA, and nonsilent mutations in the translated regions of the genome, are listed). The p35 sequence was determined by Cohen et al. (1987b).

d - Deletion

Table 3. Cytopathic Effect and Intracellular and Extracellular HAV Antigen Accumulation Following Infection of FRhK-4 Cells with Cytopathic HM175 Virus Variants at Low Multiplicities*.

Virus	Cytopathic Effect (day PI)						HAV Antigen (cpm)		
	0	2	4	6	7	9	Day PI	Cells	Media
p16 HM175	0	0	0	0	0	0	9	454	76
43c	0	0	0	+(+)	++	nd	7	2145	72
18f	0	0	0	(+)	+	nd	7	3623	820

*Parallel cultures of FRhK-4 cells were established in 25 cm² flasks, and inoculated with 580 RFU 43c virus, 460 RFU 18f virus, or 1500 RFU p16 virus. Cytopathic effects were scored as: 0 = none, + = 10% cells detached, ++ = 25% cells detached; PI = postinoculation, nd = not done.

Table 4. Mutations in P1 Genomic Region of Cytopathic HM175 Variants.

Virus	Nucleotide		Amino Acid	
	Position	Substitution*	Residue	Replacement*
p16 HM175	964	A → G	2-054**	K → R
	1742	G → A		
43c	964	A → G	2-054	K → R
	1678	A → C	3-070	D → A
	1742	G → A		
	2797	A → G	1-197	N → S
	3033	A → G	1-276	M → V
18f	964	A → G	2-054	K → R
	1741	C → A	3-091	T → K
	1742	G → A		
	2684	C → U		
	3018	U → C	1-271	S → P

*From wild-type sequence (Cohen et al., 1987a).

**By convention, residue "2-054" is residue 54 of capsid protein VP2.

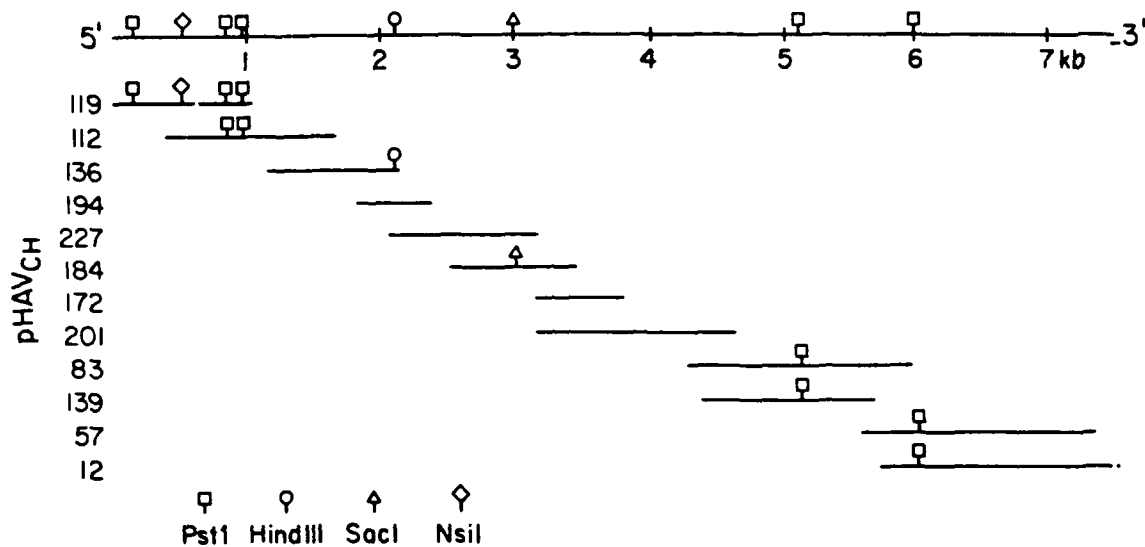


Figure 1. Physical map of pHAV_{CH} clones containing inserts of p16 HM175 cDNA.

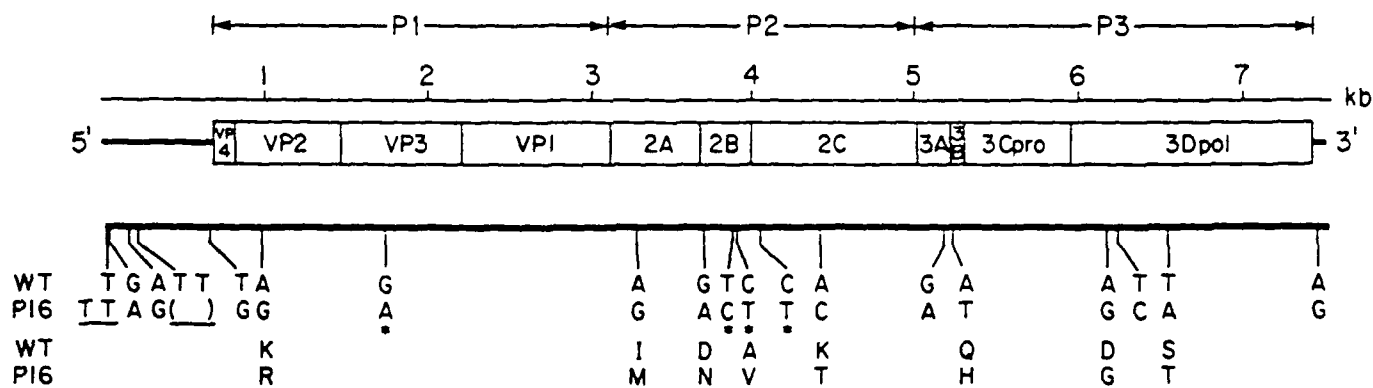


Figure 2. Genomic location of mutations present in p16 HML75 virus. Putative protein assignments are as described by Cohen et al. (1987a). The sequence of p16 HML75 virus was compared with that reported for wild-type HML75 (3 marmoset passages).

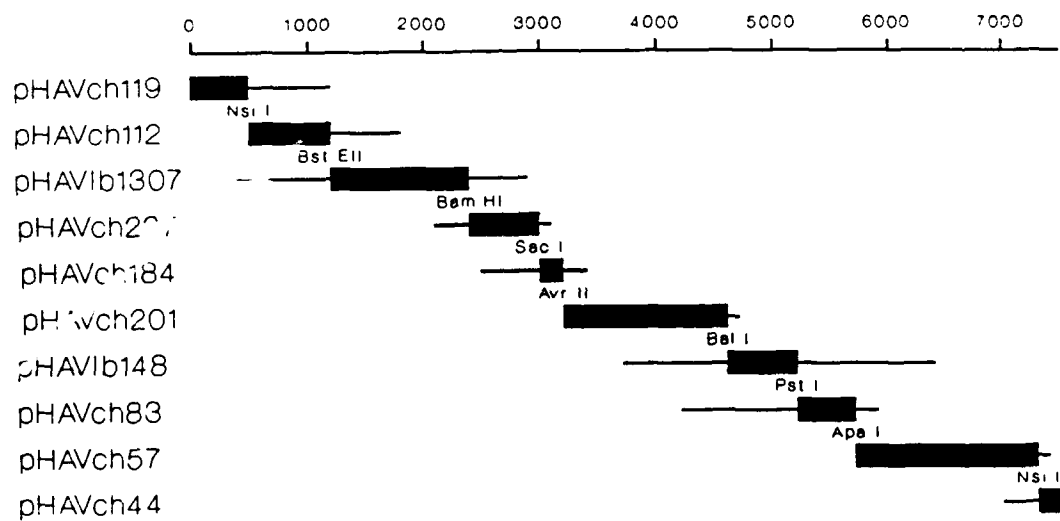


Figure 3. Map of the genomic-length p16 Hm175 construct pHAV_{ch}p16. Restriction sites employed for assembly of the construct and the contributing p16 Hm175 cDNA plasmid clones are shown.

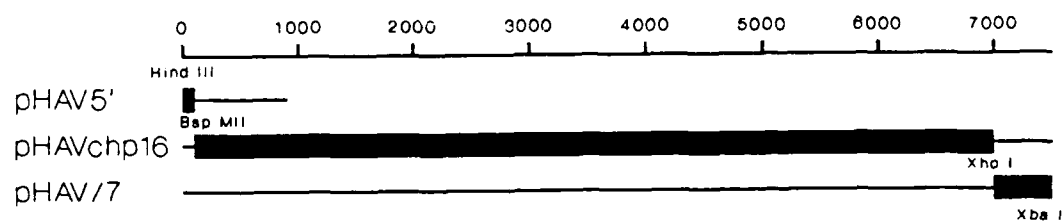


Figure 4. Final construction of pHAV/p16: ligation of fragments from pHAV5', pHAV_{chp16}, and pHAV/7 to create a genomic-length p16 HM175 sequence inserted between HindIII and XbaI sites of the plasmid vector pGEM3.

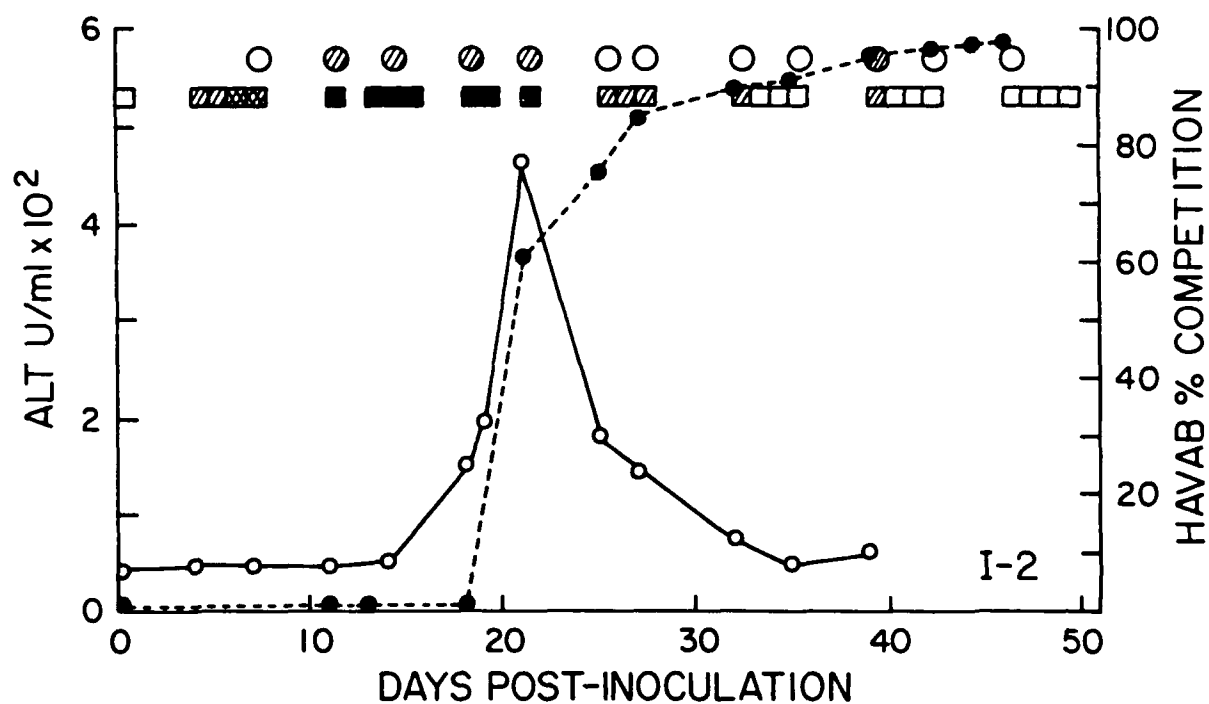


Figure 5. Infection of a seronegative owl monkey (*Aotus trivirgatus*) with pl HM175/S18 virus. Serum ALT activity and anti-HAV detected by radio-immunoassay (HAVAB) are shown: greater than 50% inhibition in the radio-immunoassay indicates the presence of antibody.

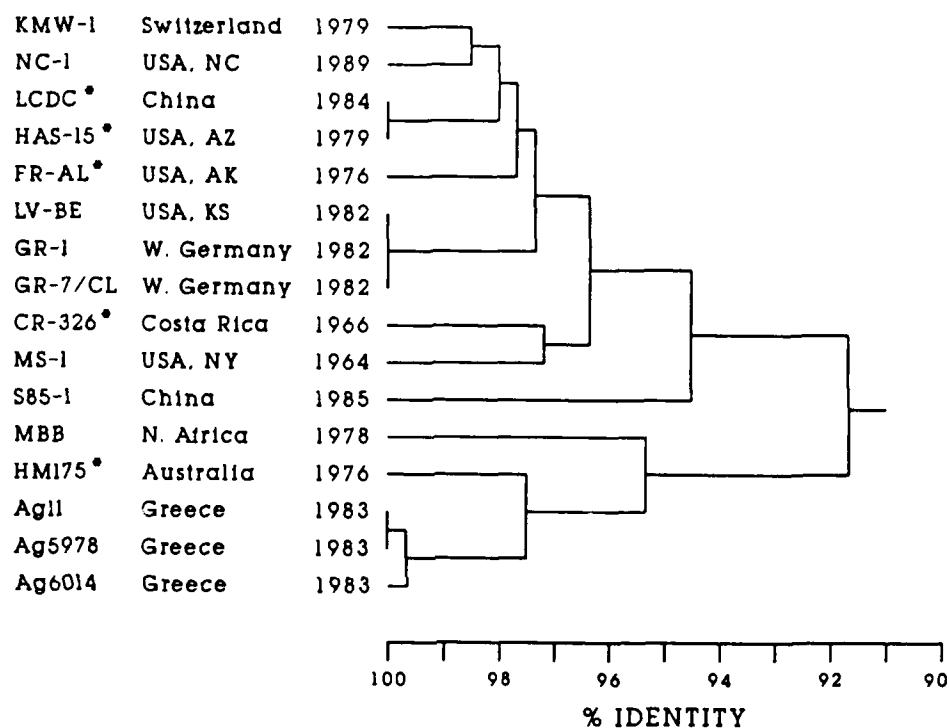


Figure 6. Dendrogram showing relatedness between nucleotide sequences of HAV strains from epidemiologically diverse sources. Differences between colinear sequences spanning bases 2056 to 2208 and 3020 to 3191 (325 total) were combined for this analysis; similar results were independently obtained in both genomic regions. The approximate degree of nucleotide identity between any two strains is represented by the distance from the left of the diagram to the first common node. The dendrogram was constructed by comparative subset averaging of percent identity data. (* from cell culture)


```

-----A-----T-T-A-A-A-----T-G-----T-C-----T-A-----CA-----A-T-G-----A-----T-AG-----
2208 GTTGGGATGATCTGGAGGCTTCTACCACTGTTTCAACAAAACAGATGTTCCAGACCTCAAGTTGGCATTACAACAGTGAAGGATCTTAAAGGTAGAGCAAACCAAGGAAAATG
V G D D S G G F S T T V S T K Q N V P D P Q V G I T T V K D L K G R A N Q G K H
-----E-----M-----K-----R-----

-----A-AG-A-----G-----A-A-----A-----A-T-T-----A-----A-C-----A-A
2328 GATGTTTCGGGTATCCAAGCTCCTGTAGGAGCTATCACTACCAATTGAGGATCCAGTTTGGCAAAGAAAGTGCCTGAGACCTTCCAGAAATGAAGCCTGGAGAGTCAAGACATACTTCT
D V S G I Q A P V G A I T T I E D P V L A K K V P E T F P E L K P G E S R H T S
-----V-----

-----C-C-----G-----A-G-----G-C-T-----C-----A-----T-----A-----A-C-----T-----C-----
2448 GATCATATGCTATTACAAATTTATGGGCAGATCTCATTCTTATGTACATTACATTTAATCTAATAACAAAGAGTACACTTTTCTATCACTTTGTCACTCAACTTCTAATCCTCCT
D H M S I Y K F M G R S H F L C T F T F N S N N K E Y T F P I T L S S T S M P P

-----T-----A-----A-----G-----C-----T-G-----T-G-----A-G-----T-A-----C-----T-----A-----A-----A-----C-----C-----C-----
2568 CATGGATTGCTTCAACTCTGAGATGGTTTTTAACTTTTTCAGCTTTATAGGGTCCCTTGGATTGACAATAATATAACTGGGGTACTGATGTTGATGGAATGGCTTGGTTTACT
H G L P S T L R W F F N L F Q L Y R G P L D L T I I I T G A T D V D G M A W F T

-----A-A-TC-T-C-T-----T-T-----A-----A-T-G-----C-----A-----T-----A-----C-----A-----G-----G-----
2688 CCCGTTGGGTAGCAGTAGATACCCCATGGGTTGAGAAGGAGTCTGCTCTTCTATTGATTACAAGACAGCTCTTGGTGTCTTAGGTTAATACTAGAAGAACAGGAAACATTGAGATT
P V G L A V D T P W V E K E S A L S I D Y K T A L G A V R F N T R R T G N I Q I

-----A-A-A-----T-T-----T-A-----G-----T-A-----TT-G-----G-----T-A-----T-G-----T-----G-----T-----T-----
2808 AGGTGCGCTGGTACTCCTATCTTTATGCTGTCTCAGGGGCACTGGATGGGCTGGAGACAAAACAGATTCAACTTTGGACTTGTCTCCATTCAAAATGCAAAATTACAATCACTCAGAT
R L P W Y S Y L Y A V S G A L D G L G D K T D S T F G L V S I Q I A N Y N H S D

-----C-----T-----C-----A-----A-----A-----C-----T-----A-----CT-A-----C-----T-----A-----CA-1-----T-----AGC-----A
2928 GAATATTTGCTTTTAGTGTACTTGTCTGTGACTGAACAGTCTGAGTTTATTTTCTAGAGCACCTTTGAATACCAATGCTATGATGTCATCAGAAACAAATGATGGATAGAATTGCT
E Y L S F S C Y L S V T E Q S E F Y F P R A P L N T N A M M S S E T M M D R I A
-----S-----L-----T-----S-----S-----

GC---A---CT-G---G---A-----G-----A---T-A---G---A---AAA-G-----G
3048 CTTGGTGATCTTGAATCCTCAGTTGATGATCCTCGAACTGAAGAGGATCGTAAATTTGAA
L G D L E S S V D D P R T E E D R K F E
A - - - - - S - - - K R - -

```

Figure 7. Nucleotide and predicted amino acid sequence of the PA21 capsid protein VP1. Numbering and cleavage sites are based on those of Cohen et al., 1987a. The differences present in the HM175 sequence are displayed above (nucleotides) or below (amino acids) the PA21 sequence.

LITERATURE CITED

- ANDERSON, D.A. (1987) Cytopathology, plaque assay, and heat inactivation of hepatitis A virus strain Hm175. *J. Med. Virol.* 22, 35-44.
- ANDERSON, D.A., LOCARNINI, S.A., ROSS, B.C., COULEPIS, A.G., ANDERSON, B.N., and GUST, I.D. (1987) Single-cycle growth kinetics of hepatitis A virus in BSC-1 cells, p. 497-507. In Positive strand RNA viruses, M.A. Brinton and R.R. Rueckert (eds.). Alan R. Liss, Inc., New York City.
- ANDERSON, D.A., ROSS, B.C., and LOCARNINI, S.A. (1988) Restricted replication of hepatitis A virus in cell culture: encapsidation of viral RNA depletes the pool of RNA available for replication. *J. Virol.* 62, 4201-4206.
- ANDREWS, N.C., and BALTIMORE, D. (1986) Purification of a terminal uridylyl-transferase that acts as host factor in the in vitro poliovirus replicase reaction. *Proc. Nat. Acad. Sci. USA* 83, 221-225.
- BINN, L.N., BANCROFT, W.H., LEMON, S.M., MARCHWICKI, R.H., LEDUC, J.W., TRAHAN, C.J., STALEY, E.C., and KEENAN, C.M. (1986) Preparation of a prototype inactivated hepatitis A virus vaccine from infected cell cultures. *J. Infect. Dis.* 153, 749-756.
- BINN, L.N., LEMON, S.M., MARCHWICKI, R.H., REDFIELD, R.R., GATES, N.L., and BANCROFT, W.H. (1984) Primary isolation and serial passage of hepatitis A virus strains in primate cell cultures. *J. Clin. Microbiol.* 20, 28-33.
- BOGGS, J.D., MELNICK, J.L., CONRAD, M.E. and FELSHER, B.F. (1970) Viral hepatitis: clinical and tissue culture studies. *JAMA* 214, 1041-1046.
- BROWN, E., JANSEN, R.W., and LEMON, S.M. (1989) Characterization of a Simian Hepatitis A Virus (HAV): Antigenic and Genetic Comparison with Human HAV. *J. Virol.* (submitted).
- CANN, A.J., STANWAY, G., HAUPTMAN, R., MINOR, P.D., SCHILD, G.C., CLARKE, L.D., MOUNTFORD, R.C., and ALMOND, J.W. (1983) Poliovirus type 3: molecular cloning of the genome and nucleotide sequence of the region encoding the protease and polymerase proteins. *Nucleic Acids Res.* 11, 1267-1281.
- COHEN, J.I., TICEHURST, J.R., PURCELL, R.H., BUCKLER-WHITE, A., and BAROUDY, B.M. (1987a) Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. *J. Virol.* 61, 50-59.
- COHEN, J.I., ROSENBLUM, B., TICEHURST, J.R., DAEMER, R.J., FEINSTONE, S.M., and PURCELL, R.H. (1987b) Complete nucleotide sequence of an attenuated hepatitis virus: comparison with wild-type virus. *Proc. Nat. Acad. Sci. USA* 84, 2497-2501.
- COHEN, J.I., TICEHURST, J.R., FEINSTONE, S.M., ROSENBLUM, B., and PURCELL, R.H. (1987c) Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. *J. Virol.* 61, 3035-3039.

- CROMEANS, T., FIELDS, H.A., and SOBSEY, M.D. (1989) Replication kinetics and cytopathic effect of hepatitis A virus. *J.Gen.Virol.*
- CROMEANS, T., SOBSEY, M.D., and FIELDS, H.A. (1987) Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. *J.Med.Virol.* 22, 45-56.
- DAEMER, R.J., FEINSTONE, S.M., GUST, I.D., and PURCELL, R.H. (1981) Propagation of human hepatitis A virus in African Green Monkey kidney cell culture: primary isolation and serial passage. *Infect.Immun.* 32, 388-393.
- FRÖSNER, G.G., DEINHARDT, F., SCHEID, R., GAUSS-MÜLLER, V., HOLMES, N., MESSELBERGER, V., SIEGL, G., and ALEXANDER, J.J. (1979) Propagation of human hepatitis A virus in a hepatoma cell line. *Infection* 7, 303-305.
- GAUSS-MÜLLER, V., and DEINHARDT, F. (1984) Effect of hepatitis A virus infection on cell metabolism *in vitro*. *Proc.Soc.Exper.Biol.Med.* 175, 10-15.
- GUST, I.D., LEHMANN, N.I., CROWE, S., MCCRORIE, M., LOCARNINI, S.A., and LUCAS, C.R. (1985) The origin of the HMI75 strain of hepatitis A virus. *J. Infect. Dis.* 151, 365-367.
- JANSEN, R.W., NEWBOLD, J.E., and LEMON, S.M. (1985) Combined immunoaffinity cDNA-RNA hybridization assay for detection of hepatitis A virus in clinical specimens. *J. Clin. Microbiol.* 22, 984-989.
- JANSEN, R.W., NEWBOLD, J.E., and LEMON, S.M. (1987) Mutations in the capsid-encoding and 5' nontranslated regions of the hepatitis A virus genome associated with adaptation of virus to growth in cell culture. Viral Hepatitis and Liver Disease, Alan R. Liss, Inc. New York. pp. 36-42.
- JANSEN, R.W., NEWBOLD, J.E., and LEMON, S.M. (1988) Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for *in vitro* replication. *Virology* 163, 299-307.
- JANSEN, R.W., and LEMON, S.M. (1989) Molecular epidemiology of hepatitis A virus defined by an antigen-capture/polymerase chain reaction method. (Submitted for publication).
- KARRON, R.A., DAEMER, R.J., TICEHURST, J.R., et al (1988) Studies of prototype live hepatitis A virus vaccine in primate models. *J.Infect.Dis.* 157, 338-345.
- KRUGMAN, S., GILES, J.P., and HAMMOND, J. (1967) Infectious hepatitis: evidence for two distinctive clinical, epidemiological, and immunological types of infection. *JAMA* 200, 365-373.
- LEDNAR, W.M., LEMON, S.M., KIRKPATRICK, J.W., REDFIELD, R.R., FIELDS, M.L. and KELLEY, P.W. (1985) Frequency of illness associated with epidemic hepatitis A virus infections in adults. *Am. J. Epidemiol.* 122, 226-233.

- LEDUC, J.W., LEMON, S.M., KEENAN, C.M., GRAHAM, R.R., MARCHWICKI, R.H., and BINN, L.N. (1983) Experimental infection of the New World owl monkey (*Aotus trivirgatus*) with hepatitis A virus. *Infect. Immun.* 40, 766-772.
- LEMON, S.M., LEDUC, J.W., BINN, L.N., ESCAJADILLO, A., and ISHAK, K.G. (1982) Transmission of hepatitis A virus among recently captured Panamanian owl monkeys. *J. Med. Virol.* 10, 25-36.
- LEMON, S.M., MARCHWICKI, R.H. and BINN, L.N. (1983) Radioimmunofocus assay for quantitation of hepatitis A virus in cell culture. *J. Clin. Microbiol.* 17, 834-839.
- LEMON, S.M., and BINN, L.N. (1983a) Antigenic relatedness of two strains of hepatitis A virus determined in a virus neutralization assay. *Infect. Immun.* 42, 418-420.
- LEMON, S.M., and BINN, L.N. (1983b) Serum neutralizing antibody response to hepatitis A virus. *J. Infect. Dis.* 148, 1033-1039.
- LEMON, S.M. (1985) Type A viral hepatitis: new developments in an old disease. *N. Engl. J. Med.* 313, 1059-1067.
- LEMON, S.M., and BINN, L.N. (1985) Incomplete neutralization of hepatitis A virus *in vitro* due to lipid-associated virions. *J. Gen. Virol* 66, 2501-2505.
- LEMON, S.M., and JANSEN, R.W. (1985) A simple method for clonal selection of hepatitis A virus based on recovery of virus from radioimmunofocus overlays. *J. Virol. Meth.* 11, 171-176.
- LEMON, S.M., STAPLETON, J.T., LEDUC, J.W., TAYLOR, D., MARCHWICKI, R., and BINN, L.N. (1987a) Cell-culture-adapted variant of hepatitis A virus selected for resistance to neutralizing monoclonal antibody retains virulence in owl monkeys. Viral Hepatitis and Liver Disease, Alan R. Liss, Inc. New York. pp. 70-73
- LEMON, S. M, CHAO, S.-F., JANSEN, R.W., BINN, L.N., and LEDUC, J.W. (1987b) Genomic heterogeneity among human and nonhuman strains of hepatitis A virus. *J Virol* 61, 735-742.
- LEMON, S.M., BINN, L.N., MARCHWICKI, R., MURPHY, P.C., PING, L.-H., JANSEN, R.W., ASHER, L.V.S., STAPLETON, J.T., TAYLOR, D.G. and LEDUC, J.W. (1989) In vivo replication and reversion to wild-type of a neutralization-resistant antigenic variant of hepatitis A virus. *J. Inf. Dis.*, in press.
- LINEMEYER, D.L., MENKE, J.G., MARTIN-GALLARDO, A., HUGHES, J.V., YOUNG, A. and MITRA, S.W. (1985) Molecular cloning and partial sequencing of hepatitis A viral cDNA. *J. Virol.* 54, 247-255.
- LUO, M., VRIEND, G., KAMER, G., MINOR, I., ARNOLD, A., ROSSMANN, M.G., BOEGE, U., SCRABA, D.G., DUKE, G.M., and PALMENBERG, A.C. (1987) The atomic structure of mengo virus at 3.0 Å resolution. *Science* 235, 182-189.

MACGREGOR, A.W., KORNITSCHUK, M., HURREL, J.G.R., LEHMANN, N.I., COULEPIS, A.G., LOCARNINI, S.A., and GUST, I.D. (1983) Monoclonal antibodies against hepatitis A virus. *J. Clin. Microbiol.* 18, 1237-1243.

MANIATIS, T., FRITSCH, E.F., and SAMBROOK, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

MINOR, P.D., SCHILD, G.C., CANN, A.J., DUNN, G., EVANS, D.M.A., FERGUSON, M., STANWAY, G., WESTROP, G., and ALMOND, J.W. (1986) Studies on the molecular aspects of antigenic structure and virulence of poliovirus. *Ann. Inst. Pasteur/Virol.* 137 E, 107-125.

MORROW, C.D., LUBINSKI, J., HOCKO, J., GIBBONS, G.F., and DASGUPTA, A. (1985) Purification of a soluble template-dependent rhinovirus RNA polymerase and its dependence on a host cell protein for viral RNA synthesis. *J. Virol.* 53, 266-272.

NASSER, A.M., and METCALF, T.G. (1987) Production of cytopathology in FRhK-4 cells by BS-C-1-passaged hepatitis A virus. *Appl. Environ. Microbiol.* 53, 2967-2971.

NOMOTO, A., KOHARA, M., KUGE, S., KAWAMURA, N., ARITA, M., KOMATSU, T., ABE, S., SEMLER, B.L., WIMMER, E., and ITOH, H. (1987) Study on virulence of poliovirus type 1 using *in vitro* modified viruses. In: Brinton MA, Rueckert RR, eds. Positive Strand RNA Viruses. New York City: Alan R. Liss, pp 437-452.

OMATA, T., KOHARA, M., KUGE, S., KOMATSU, T., ABE, S., SEMLER, B.L., KAMEDA, A., ITOH, H., ARITA, M., WIMMER, E., and NOMOTO, A. (1986) Genetic analysis of the attenuation phenotype of poliovirus type 1. *J. Virol.* 58, 348-358.

OVCHINNIKOV, YU.A., SVERDLOV, E.D., TSAREV, S.A., ARSENJAN, S.G., ROHLINA, T.O., CHIZHIKOV, V.E., PETROV, N.A., PRIHODJKO, G.G., BLINOV, V.M., VASILENKO, S.K., KUSOV, YU.YU., GRABKO, V.I., FLEER, G.P., BALAYAN, M.S. and DROZDOV, S.G. (1985) Sequence of 3372 nucleotides of a hepatitis A virus (HAV) strain cDNA coding for VP4-VP1 and non-structural proteins: comparison of various HAV strains. *Biokhimiya* 285, 1014-1018.

PAUL, A.V., TADA, H., VON DER HELM, K., WISSEL, T., KIEHN, R., WIMMER, E. and DEINHARDT, F. (1987) The entire nucleotide sequence of the genome of human hepatitis A virus (isolate MBB). *Virus Res.* 8, 153-171.

PELLETIER, J., and SONENBERG, N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature.* 334, 320-325.

PINCUS, S.E., DIAMOND, D.C., EMINI, E.A., and WIMMER, E. (1986) Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. *J. Virol.* 57, 638-646.

PING, L.-H., JANSEN, R.W., STAPLETON, J.T., COHEN, J.I., and LEMON, S.M. (1988) Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus. *Proc. Natl. Acad. Sci. USA* 85, 8281-8285.

- PRABHAKAR, B.S., SRINIVASAPPA, J., and RAY, U. (1987) Selection of coxsackievirus B₄ variants with monoclonal antibodies results in attenuation. *J. Gen. Virol.* **69**, 865-869.
- PROVOST, P.J., and HILLEMANN, M.R. (1979) Propagation of human hepatitis A virus in cell culture *in vitro*. *Proc.Soc.Exper.Biol.Med.* **160**, 213-221.
- PROVOST, P.J., BANKER, F.S., GIESA, P.A., MCALEER, W.J., BUYNACK, E.B., and HILLEMANN, M.R. (1982) Progress toward a live, attenuated human hepatitis A vaccine. *Proc.Soc.Exper.Biol.Med.* **170**, 8-14.
- PROVOST, P.J., BISHOP, R.P., GERETY, R.J., HILLEMANN, M.R., MCALEER, W.J., SCOLNICK, E.M., and STEVENS, C.E. (1986) New findings in live, attenuated hepatitis A vaccine development. *J. Med. Virol.* **20**, 165-175.
- PURCELL, R.H. (1987) Status of hepatitis A vaccines, p. 565-574. In Positive strand RNA viruses. Brinton, M.A., and Rueckert, R.R. (eds.). Alan R. Liss, Inc., New York City.
- RAKELA, J., FAY, O.H., STEVENSON, D., GORDON, I., and MOSLEY, J.W. (1976) Similarities of two hepatitis A virus strains. *Bull.WHO* **54**, 561-564.
- RICO-HESSÉ, R., PALLANSCH, M.A., NOTTAY, B.K. AND KEW, O.M. (1987) Geographic distribution of wild poliovirus type 1 genotypes. *Virology* **160**, 311-322.
- ROSS, B.C., ANDERSON, B.N., COULEPIS, A.G., CHENOWETH, M.P., and GUST, I.D. (1986) Molecular cloning of cDNA from hepatitis A virus strain HM-175 after multiple passages *in vivo* and *in vitro*. *J. Gen. Virol.* **67**, 1741-1744.
- SABIN, A.B. (1985) Oral poliovirus vaccine: history of its development and use and current challenge to eliminate poliomyelitis from the world. *J. Inf. Dis.* **151**, 420-436.
- SAIKI, R.K., GELFAND, D.H., STOFFEL, S., SCHARF, S.J., HIGUCHI, R., HORN, G.T., MULLIS, K.B. and ERLICH, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.
- SANGER, R., NICKLEN, S., and COULSON, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.
- SIEGL, G., WEITZ, M., and KRONAUER, G. (1984) Stability of hepatitis A virus. *Intervirology* **22**, 218-226.
- STANWAY, G., CANN, A.J., HAUPTMAN, R., HUGHES, P., and ALMOND, J.W. (1983) The nucleotide sequence of poliovirus type 3 Leon a₁b: comparison with poliovirus type 1. *Nucleic Acids Res.* **11**, 5629-5643.
- STAPLETON, J.T., and LEMON, S.M. (1987) Neutralization escape mutants define dominant immunogenic neutralization site on hepatitis A virus. *J. Virol.* **61**, 491-498.

TICEHURST, J.R., RACANIELLO, V.R., BAROUDY, B.M., BALTIMORE, D., PURCELL, R.H., and FEINSTONE, S.M. (1983) Molecular cloning and characterization of hepatitis A virus cDNA. *Proc. Nat. Acad. Sci. USA* **80**, 5885-5889.

TICEHURST, J.R., COHEN, J.I., FEINSTONE, S.M., PURCELL, R.H., JANSEN, R.W. and LEMON, S.M. (1989) Replication of hepatitis A virus: new ideas from studies with cloned cDNA. In *Molecular Aspects of Picornavirus Infection and Detection*, eds. Ehrenfeld, E. and Semler, B.L. (ASM Press, Washington, DC), pp. 27-50.

VALLBRACHT, A., HOFMANN, L., WURSTER, K.G., and FLEHMIG, B. (1984) Persistent infection of human fibroblasts by hepatitis A virus. *J.Gen.Virol.* **65**, 609-615.

VENUTI, A., DI RUSSO, C., DEL GROSSO, N., et al (1985) Isolation and molecular cloning of a fast-growing strain of human hepatitis A virus from its double-stranded replicative form. *J.Virol.* **56**, 579-588.

WEITZ, M. AND SIEGL, G. (1985) Variation among hepatitis A virus strains. I. Genomic variation detected by T₁ oligonucleotide mapping. *Virus Res.* **4**, 53-67.

WEITZ, M., BAROUDY, B.M., MALOY, W.L., TICEHURST, J.R., and PURCELL, R.H. (1986) Detection of a genome-linked protein (VPg) of hepatitis A virus and its comparison with other picornaviral VPgs. *J. Virol.* **60**, 124-130.

WHEELER, C.M., FIELDS, H.A., SCHABLE, C.A., MEINKE, W.J., and MAYNARD, J.E. (1986) Adsorption, purification, and growth characteristics of hepatitis A virus strain HAS-15 propagated in fetal rhesus monkey kidney cells. *J.Clin.Microbiol.* **23**, 434-440.

PUBLICATIONS SUPPORTED BY THIS CONTRACT

1. STAPLETON, J.T., and LEMON, S.M. (1987). Neutralization escape mutants define dominant immunogenic neutralization site on hepatitis A virus. *J. Virol.* 61, 491-498.
2. JANSEN, R.W., NEWBOLD, J.E., and LEMON, S.M. (1988). Mutations in the capsid-encoding and 5' nontranslated regions of the hepatitis A virus genome associated with adaptation of virus to growth in cell culture. Viral Hepatitis and Liver Disease, Alan R. Liss, Inc. New York. pp. 36-42.
3. LEMON, S.M., STAPLETON, J.T., LEDUC, J.W., TAYLOR, D., MARCHWICKI, R., and BINN, L.N. (1988). Cell-culture-adapted variant of hepatitis A virus selected for resistance to neutralizing monoclonal antibody retains virulence in owl monkeys. Viral Hepatitis and Liver Disease, Alan R. Liss, Inc. New York. pp. 70-73
4. LEMON, S.M., CHAO, S.-F., JANSEN, R.W., BINN, L.N., and LEDUC, J.W. (1987). Genomic heterogeneity among human and nonhuman strains of hepatitis A virus. *J. Virol.* 61, 735-742.
5. JANSEN, R.W., NEWBOLD, J.E., and LEMON, S.M. (1988). Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for *in vitro* replication. *Virology* 163, 299-307.
6. LEMON, S.M., CHAO, S.-F., and R.W. JANSEN. (1988). Low sequence homology between P1-P2 region RNA from human and primate strains of hepatitis A virus. Proceeding of the LSU-International Symposium on Viral Hepatitis and AIDS.
7. PING, L.-H., JANSEN, R.W., STAPLETON, J.T., COHEN, J.I., and LEMON, S.M. (1988). Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus. *Proc.Natl.Acad.Sci.USA* 85, 8281-8285.
8. LEMON, S.M., PING, L.-H., MURPHY, P., DAY, S.P., and JANSEN, R.W. (1989) Characterization of an immunodominant antigenic site of hepatitis A virus. R. Chanock, F. Brown, R. Lerner and H. Ginsberg (eds). *Vaccines 89, Modern Approaches to New Vaccines Including Prevention of AIDS*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
9. LEMON, S.M., BINN, L.N., MARCHWICKI, R., MURPHY, P.C., PING, L.-H., JANSEN, R.W., ASHER, L.V.S., STAPLETON, J.T., TAYLOR, D.G. and LEDUC, J.W. (1989) *In vivo* replication and reversion to wild-type of a neutralization-resistant antigenic variant of hepatitis A virus. *J. Inf. Dis.*, in press.
10. LEMON, S.M., and PING, L.-H. (1989) Antigenic structure of hepatitis A virus. In *Molecular Aspects of Picornavirus Infection and Detection*,

eds. Semler, B.L., and Ehrenfeld, E. (ASM Press, Washington, DC), pp. 193-208.

11. TICEHURST, J.R., COHEN, J.I., FEINSTONE, S.M., PURCELL, R.H., JANSEN, R.W. and LEMON, S.M. (1989). Replication of hepatitis A virus: new ideas from studies with cloned cDNA. In *Molecular Aspects of Picornavirus Infection and Detection*, eds. eds. Semler, B.L., and Ehrenfeld, E. (ASM Press, Washington, DC), pp. 27-50.
12. BROWN, E., JANSEN, R.W., and LEMON, S.M. (1989) Characterization of a Simian Hepatitis A Virus (HAV): Antigenic and Genetic Comparison with Human HAV. *J. Virol.* (in press).

PERSONNEL RECEIVING CONTRACT SUPPORT

Stanley M. Lemon, M.D.	(Principal Investigator)
John E. Newbold, Ph.D.	(Coinvestigator)
Li-Hua Ping, M.D.	(Visiting Research Scientist)
Stephen Day, Ph.D.	(Research Associate)
Robert W. Jansen, M.S.	(Research Analyst)
Paula C. Murphy, B.S.	(Research Technician III)